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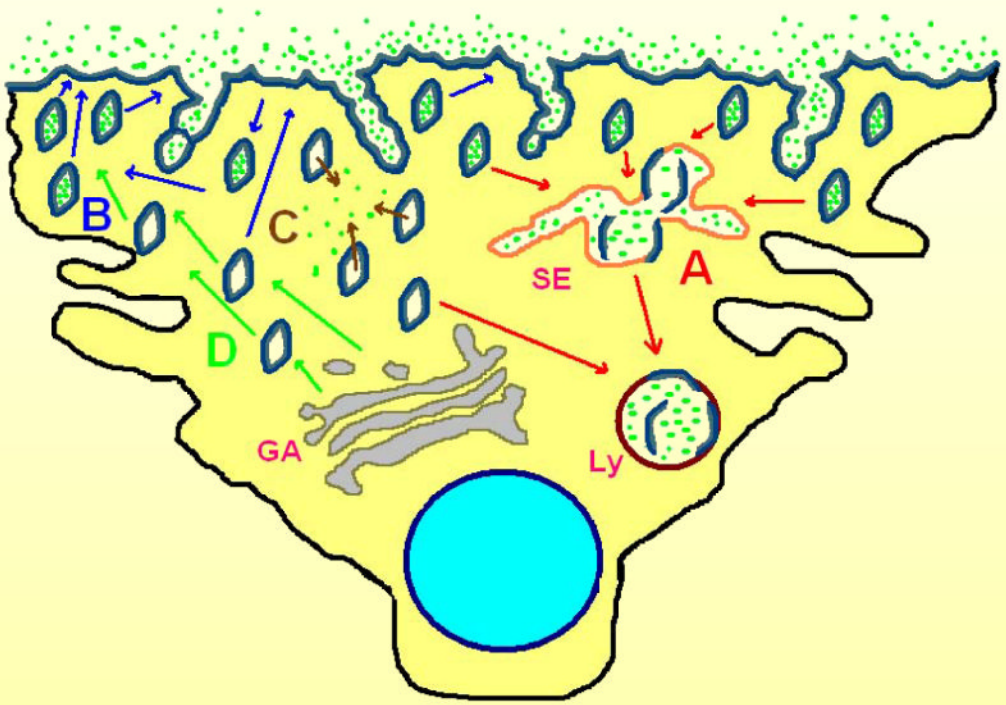
ENDOCYTOSIS

STRUCTURAL COMPONENTS, FUNCTIONS AND PATHWAYS



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CONTENTS

PREFACE

Endocytosis is the process by which cells absorb molecules (such as proteins) from outside the cell by engulfing it with their cell membrane. It is used by all cells of the body because most substances important to them are large polar molecules that cannot pass through the hydrophobic plasma membrane or cell membrane. Endocytosis is required for a vast number of functions that are essential for the well being of a cell. It intimately regulates many processes, including nutrient uptake, cell adhesion and migration, receptor signaling pathogen entry, neurotransmission, receptor downregulation, antigen presentation, cell polarity, mitosis, growth and differentiation, and drug delivery. This new book examines the functions and benefits of endocytosis in regards to cells within the body.

Chapter 1 - Alzheimer Amyloid Precursor Protein (APP) is a ubiquitously expressed transmembrane protein that is involved in the pathogenesis of Alzheimer's disease. Cellular trafficking of APP is mediated by several targeting signals in its cytoplasmic tail, which are responsible for the interaction with the targeting machineries. APP undergoes several proteolytic processing steps which are carried out by different enzymes. The pathological amyloid β-peptide is generated as a result of the sequential action of two proteases termed β- and γ- secretases. For the processing of APP by the β-secretase, endocytosis of APP from the plasma membrane is necessary, and the processing takes place in endosomal compartments. Recent findings have suggested that the cholesterol and sphingolipid rich membrane microdomains known as rafts would be important players in the regulation of the pathological processing of APP. In this chapter, we will focus on describing the targeting signals in APP that mediate its transport into different cellular organelles and domains by means of interaction with the targeting machineries. In addition, we will especially summarize the recent findings implicating the role of rafts in the proteolytic processing of APP and in the pathogenesis of Alzheimer's disease.

Chapter 2 - The Na/K-ATPase was discovered as an energy transducing ion pump. A major difference between the Na/K-ATPase and other P-type ATPases is its ability to bind a group of chemicals called cardiotonic steroids (CTS). Endogenous CTS have been identified as a new class of endogenous hormones, functioning as important regulators of renal Na^+ excretion and blood pressure. Na/K-ATPase is not only an ion pump, but also an important receptor that can transduce ligand-like effect of CTS on intracellular protein kinases. Significantly, the CTS-provoked kinase cascades are capable of inducing endocytosis of the apical NHE3 (Na/H exchanger isoform 3) and basolateral Na/K-ATPase in renal proximal tubular cells. Functionally, this CTS-induced coordinately regulation of Na/K-ATPase and

NHE3 leads to the inhibition of sodium reabsorption in renal proximal tubules under physiological conditions, such as high salt diet. A defect in this regulation would reduce the ability of renal proximal tubular cells to excrete Na^+, thus a contributor of salt-sensitive hypertension.

Chapter 3 - Endocytosis is defined as a process where patches of membrane are invaginated and budded off of specialized domains of the plasma membrane. The receptor-mediated endocytosis or clathrin dependant is the most studied mechanism, followed by caveolar and non-clathrin dependant. But in diverse type of cells, such as umbrella cells from the uroepithelium, changes in plasma membrane tension are capable to induce endocytosis. This process occurs by hydrostatic pressure changes during micturion cycle, is clathrin independant and an intact cytoskeleton is necessary. When hydrostatic pressure increases, during urinary bladder filling-phase of micturition cycle, the umbrella cells are stretched and a fusion process of subapical vesicles to apical plasma membrane occurs. After plasma membrane relaxation, during the voiding-phase of the cycle, the additional membrane is endocytosed as discoidal vesicles entrapping the luminal fluid phase. This peculiar endocytosis/exocytosis process, different from the classical endocytosis mechanism, result an appropriate membrane model to study the membrane traffic but also the urinary fluid traffic. The late may have physiophatological implications since the toxins present in the urinary fluid may effectively be delivered to the cell machinery instead to be recycled to the bladder lumen.

Chapter 4 - Cells use various mechanisms to internalize substances presented in their surroundings, with pinocytosis, phagocytosis, macropinocytosis, caveolae/raft-dependent endocytosis, and receptor-mediated clathrin-dependent endocytosis being the most extensively studied. Pinocytosis occurs independently of actin polymerization. In contrast, the importance of F-actin remodeling during internalization by the other mechanisms is well established. Similarly, the key role of membrane-proximal tyrosine kinases during internalization of small receptor ligands such as growth factors, or much larger bacteria, is undeniable. Surprisingly though, the molecular mechanisms linking tyrosine kinase activation and cytoskeletal reorganization pathways to endocytosis are not completely understood. cAbl is a member of the only family of tyrosine kinases known to date to have an actin-binding domain that allows them to interact with both globular and filamentous actin [1-7] and yet, our understanding of its role in endocytosis is still in its infancy. Herein, I review the evidence supporting a role for cAbl in the uptake of ligated receptors, apoptotic cells, bacteria and viruses. Specifically, I describe and discuss cAbl-dependent pathways involved in 1) clathrin-mediated endocytosis of two different receptors (EGFR and BCR), 2) caveolin-dependent endocytosis of viruses, and 3) macropinocytosis of bacteria and apoptotic cells, highlighting the overlap and differences between them. Finally, I discuss the potential significance of having specific endocytic mechanisms adapted to given conditions, as well as the significance of cAbl's role in endocytosis linked to physiological signal transduction and pathological conditions such as cancer and fibrosis.

Chapter 5 - In this review, the cellular uptake mechanism of inorganic nanoparticles, as drug delivery carrier, is discussed, particularly focusing on layered double hydroxide (LDH). The LDH nanoparticles are biocompatible in general consisting of positively charged metal hydroxide sheets and charge-compensating interlayer anions, which are solvated with water or solvent molecules depending upon synthetic conditions. Such inorganic nanoparticles have attracted a great deal of attention nowadays as nanovehicles due to their efficient

delivery behaviors for drugs or genes into cells. Specific internalization pathway, cellular uptake rate, and delivery efficiency of LDHs will be described in details with respect to their structural features such as particle size (50, 100, 200 and 350 nm) and surface modification (folate conjugates). Cellular entry of other inorganic nanoparticles including carbon nanotube, iron oxide and silica will be also discussed with LDHs comparatively. And finally attempts will be made to understand the interaction mechanism between delivery nanovehicles and cells, because it will provide a new perspective for the design of drug delivery nanovehicles with maximized and targeted delivery ability.

Chapter 6 - The small GTPase Rho and Rho-associated protein kinase (ROCK) signaling pathway has been demonstrated to be one of the major pathways involved in tumor invasion through reorganization of actin cytoskeleton. It was shown previously that an expression of constitutively active form of RhoA or of ROCK in rat hepatoma cells considerably promoted invasive ability of these cells in vitro and in vivo, and enhanced phosphorylation level of myosin light chain MLC20, thereby, indicating that Rho-ROCK pathway mediates tumor cell motility and invasion. ROCK can phosphorylate and activate LIM kinase 1 (LIMK1), which leads to phosphorylation of cofilin. LIMK 1 is known to play a critical role in actin cytoskeletal remodeling by linking the signal from the Rho family of small GTPases to the change in cofilin activity, thereby, indicating an important role for Rho-ROCK-LIMK1-cofilin signaling in tumor cell invasion through regulating actin dynamics. We reported previously that overexpression of LIMK1 resulted in a marked retardation of EGF receptor endocytosis in low-invasive human breast cancer cell MCF-7. Thereby, we postulate that LIMK 1 signaling plays an important role in the regulation of ligand-induced endocytosis of EGF receptor in tumor cells by reorganizing and influencing actin-filament dynamics. In the present study, we further assessed the effect of wild-type LIMK1, a kinase-deficient dominant negative mutant of LIMK1 (DN-LIMK1) and an active, unphosphorylatable cofilin mutant (S3A cofilin) on internalization of EGF-EGF receptor in MDA-MB-231, a highly invasive human breast cancer cell line. We demonstrate here that a marked delay in the receptor-mediated internalization of Texas red-labeled EGF was observed in the wild-type LIMK1 transfectants, and that most of the internalized EGF staining was accumulated within transferrin receptor-positive early endosomes even after 30 min internalization. In contrast, the expression of dominant-negative LIMK1 mutant rescued the efficient endocytosis of Texas red-EGF, and large amounts of Texas red-EGF staining already reached LIMPII-positive late endosomes/lysosomes after 15 min internalization. We further analyzed the effect of S3A cofilin mutant on EGF receptor endocytosis, and found an efficient delivery of Texas red-EGF into late endosomes/lysosomes at 15-30 min after internalization. Taken together, our novel findings imply that LIMK1-cofilin signaling indeed plays a pivotal role in the regulation of EGF receptor endocytosis via the early/late endocytic pathway in invasive tumor cells.

Chapter 7 - Sialic acids are a family of sugars, which typically terminate cell surface glycoconjugates. Due to this ubiquitous position, they represent key structural determinants for a number of receptors, and are potential modulators of immune responses, among which endocytosis.

Dendritic cells (DC) are professional antigen-presenting cells, crucial for linking innate and immune responses, and have been exploited as therapeutic tools to trigger immunity against tumors or pathogens, or dampen hypersensitivity responses. DCs show a wide capacity to acquire exogenous antigens through different mechanisms, which dictates its

immunoregulatory capacity. This chapter focus on the complex role of their highly sialylated cell surface content as modulator of DC endocytosis. In particular it will be presented data that support the evidence that sialylation shortage, while affecting DC maturation, results in opposing effects on macropinocytosis and phagocytosis mechanisms mediated by DCs.

Removal of the cell surface sialylated structures by sialidase treatment decreases the capacity of human monocyte-derived DC (mo-DC) to endocytose antigens, mainly uptaken by macropinocytosis, but not bacteria uptaken by phagocytosis, such as *Escherichia coli*. Sialidase treatment was found to trigger mo-DC maturation and therefore affect the cell actin cytoskeleton organization, explaining somehow macropinocytosis downregulation. Surprisingly, this treatment significantly improves the bacterial uptake by cytokine matured mo-DCs. Mouse models data suggested that the sialylated glycans mediated by ST3Gal.I and ST6Gal.I sialyltransferases are related with the observed maturation induction and subsequent decreased macropinocytosis capacity.

The complex role of sialic acid as endocytosis modulator was further enforced by findings revealing that mo-DCs express surface ectosialyltransferases, which rapidly sialylate cell surface and may interfere with mo-DC endocytosis capacity.

Of interest, the findings here described reveal a complex system involving sialylated structures that subverts part of the well known downregulation of bacteria endocytosis typical of mature mo-DC and may have a potential therapeutic application.

Chapter 8 - Endocytosis, which plays a key role in many different species, is the process that cells take up extracellular materials through plasma membranes. Protein transduction domains (PTDs), also called cell-penetrating peptides (CPPs), are small peptides and contain a large amount of basic amino acids. Several PTDs, including arginine-rich intracellular delivery (AID) peptides, were found to be responsible for cellular uptake of macromolecules. In our previous studies, AID peptides have been proven to either covalently transport proteins or noncovalently internalize proteins, DNAs or RNAs into animal or plant cells. The mechanisms by which PTD enter cells are still in vigorous debate. Our studies indicated that the possible mechanisms of AID peptide-mediated cellular entry might involve a combination of multiple internalization pathways, including at least macropinocytosis. Furthermore, our recent reports demonstrated for the first time that AID peptides could rapidly and efficiently deliver proteins into animal and plant cells in both covalent and noncovalent protein transductions (CNPT) synchronously. Therefore, investigations of cellular uptake mediated by AID peptides facilitate our understanding of endocytosis in more details and reveal nonclassically endocytic pathways.

Chapter 9 - The molecular mechanisms underlying recycling of synaptic vesicles in nerve terminals are the subject of intense investigation. In this review we consider recent progress in this field with a focus on syndapin 1, a candidate endocytic protein distinguished by its membrane-sculpting and actin remodeling properties. Syndapin 1 interacts with dynamin and the actin-regulator N-WASP, and it contains a F-BAR domain which effectively deforms membranes. In vertebrates, perturbation of syndapin 1, or of its interaction with dynamin does not influence the clathrin-dependent pathway used for synaptic vesicle recycling at moderate rates. However, synaptic vesicle recycling during high levels of synaptic activity is markedly disrupted by these treatments. This distinct role of syndapin probably reflects an involvement in bulk endocytosis, a pathway yet to be defined in molecular terms. Additionally, on the postsynaptic side, syndapin participates in clathrin-mediated uptake of a distinct set of neurotransmitter receptors during development. The synaptic role of syndapin may be limited

to vertebrates as genetic deletion of the single syndapin isoform in Drosophila does not affect synaptic function. Syndapin may thus not be regarded as a core component of the clathrin-dependent synaptic vesicle recycling machinery, because it functions in a subset of pre- and postsynaptic endocytic pathways.

Chapter 10 - Recently, a number of new methods for gene transfer have been put forward to advance biomedical study and gene therapy. Although viral vectors confer high efficiency of gene transfer, they also have the added risk of carcinogenesis, such as via random genomic integration. Although non-viral vectors lack this risk, they also tended to be less efficient for gene transfer. Thus, various efforts are being made to improve gene transfer techniques based on non-viral vectors. For example, novel chemical transfection reagents have been developed, including those with improved extracellular binding, engulfment, and utilization of the introduced nucleic acids. Additionally, receptor-mediated endocytosis has been used to create vectors using chemicals, growth factor peptides, extracellular matrix proteins, and viral proteins. This method enables selection of target cells and increased gene transfer efficiency as compared with other non-viral methods. In reviewing reports of these efforts, we have found that in terms of efficiency, differences among cells are a bigger factor than differences among transfection reagents. Some cells are always more easily transfected than others. The biological state of a given cell type, such as which endocytic pathways are functional in the cell prior to treatment, may be an important factor in endocytosis-mediated gene transfer. Therefore, studying how endocytosis is regulated should provide useful insights into how to improve gene transfer technology. Endocytosis can be initiated by the binding of ligands to their receptors on the surface of the cell membrane, followed by internalization. To make best use of this endogenous function for introduction of exogenous molecules, it will be necessary to gain a better understanding of what types of molecules efficiently induce initiation of endocytosis. In this commentary, we summarize the current state-of-the-art in non-viral gene transfer and supporting technologies related to the initiation of endocytosis. We will also discuss specific ways in which what is known about endocytosis might be exploited in order to facilitate development of new and improved non-viral methods for gene transfer**.**

Chapter 11 - Caveolae are morphologically flask-shaped invaginations of the plasma membrane identified in various types of cells such as adipocytes, endothelial cells and myocytes. Unlike clathrin-coated pits, caveolae are detergent insoluble, cholesterol- and sphingolipid-rich, and show high caveolin expression (of caveolin-1, -2 and -3). A large number of molecules have been identified to localize to the caveolae including G-protein coupled receptors, membrane receptors and small GTPases. Recent research has made it clear that caveolae can participate in endocytosis and that they are morphologically distinct from clathrin-coated pits. Caveolin-1, the constitutive protein of the caveolae, is implicated in processes of vesicular transport during caveolae-mediated endocytosis. However, the precise molecular mechanisms of caveolae-mediated endocytosis remain unclear. Rab5 is a small GTPase involved in clathrin-coated vesicle formation, vesicle-early endosome fusion, and early endosome homotypic fusion as well as endosome maturation. Rab5 cycles between the GDP- (inactive) and GTP-bound (active) forms, a process that is tightly controlled by several Rab5 associating proteins. Here we discuss how and when Rab5 activity is controlled during the caveolae-mediated endocytosis and especially focus on the crucial role of the scaffolding domain (SD domain) of cavelolin-1. We also suggest the importance of Rab5 and caveolin-1 interaction during the intracellular trafficking of caveolae on the plasma membrane to the early endosome and to one of their final destination, the Golgi apparatus.

Chapter 12 - Although the extracellular deposition of β-amyloid protein (Aβ) as senile plaques (SPs) is an invariable pathological feature of Alzheimer's disease (AD), recent studies suggest that the accumulation of intracellular Aβ may represent an early event in the pathogenesis of AD. In the case of familial AD, evidently the expression of causative genetic mutations likely enhances Aβ generation, which can cause buildup of intracellular Aβ. However, how intracellular Aβ accumulates in sporadic AD, a major form of the disease, remains to be clarified.

Recently, growing evidences suggest that endocytic dysfunction is involved in AD pathology. In brains of early stage AD patients, neuronal endocytic pathology such as the accumulation of β-amyloid precursor protein (APP) in enlarged early endosomes is observed even before SP deposition. Moreover, endocytic dysfunction induces Aβ accumulation in endosomal compartments. Aβ is produced from APP through sequential proteolytic cleavages by β- and γ-secreatases, and such amyloidogenic cleavage of APP can occur through the endocytic pathway. These findings suggest that endocytosis is involved in APP metabolism itself and that endocytic dysfunction may cause the accumulation of intracellular Aβ. In this review, I summarize findings at present and discuss the hypothesis that endocytic dysfunction may underlie AD pathology.

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Chapter 1

TRAFFICKING AND ENDOCYTOSIS OF ALZHEIMER AMYLOID PRECURSOR PROTEIN

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ABSTRACT

Alzheimer Amyloid Precursor Protein (APP) is a ubiquitously expressed transmembrane protein that is involved in the pathogenesis of Alzheimer's disease. Cellular trafficking of APP is mediated by several targeting signals in its cytoplasmic tail, which are responsible for the interaction with the targeting machineries. APP undergoes several proteolytic processing steps which are carried out by different enzymes. The pathological amyloid β-peptide is generated as a result of the sequential action of two proteases termed β- and γ- secretases. For the processing of APP by the β-secretase, endocytosis of APP from the plasma membrane is necessary, and the processing takes place in endosomal compartments. Recent findings have suggested that the cholesterol and sphingolipid rich membrane microdomains known as rafts would be important players in the regulation of the pathological processing of APP. In this chapter, we will focus on describing the targeting signals in APP that mediate its transport into different cellular organelles and domains by means of interaction with the targeting machineries. In addition, we will especially summarize the recent findings implicating the role of rafts in the proteolytic processing of APP and in the pathogenesis of Alzheimer's disease.

INTRODUCTION

Alzheimer's disease (AD) was discovered by Alois Alzheimer at the very beginning of the 20th century and is today recognized as the most common cause of dementia in elderly patients. The major causative agent of AD is the amyloid beta (Aβ or A4) peptide which is proteolytically generated from a large transmembrane precursor protein named APP for Alzheimer amyloid precursor protein. Although most cases of AD are sporadic, familial forms of the disease that result from mutations in the genes for APP or for the proteases

involved in its processing have been described. In this chapter, we aim at reviewing the most important aspects in the cell biology of APP, including its function, cellular targeting and processing by the secretases, with a special focus on the trafficking of APP in the cells and on proteins that interact with the cytoplasmic domain of APP. As the literature describing the research on APP and AD is quite overwhelming, we hereby apologize that we may not always be able to provide an exhaustive review of all studies published.

THE APP FAMILY

The amyloid precursor protein (APP, Figure 1A) was discovered in 1987 as a precursor for the 40-42 amino acid Aβ peptide. At that time, it had been suspected that the Aβ peptide might be a cleavage product originating from a bigger protein. In search for this precursor protein, Kang *et al.* constructed a cDNA library from fetal brain tissue and screened it with an Aβ probe (1). In parallel, Goldgaber *et al.* used a cDNA library from adult brain for screening (2). The isolated clones encoded a 695 amino acid protein which was found to be localized on chromosome 21.

Further investigations revealed that the APP transcript undergoes alternative splicing, leading to the expression of three different isoforms in mammalian cells, namely APP_{695}, APP_{770} and APP_{751} (3-7), all of which can serve as Aβ precursors. In mammals, APP is a member of a protein family also comprizing the "APP-like-proteins", APLP1 and APLP2, which share a high sequence similarity with each other and with APP and are also proteolytically processed (8). However, they cannot give rise to the Aβ peptide due to a lack of the Aβ domain. APLP2 and APP show largely overlapping expression patterns and are ubiquitously expressed with high abundance in the brain, while APLP1 is restricted to the nervous system (9).

PROCESSING OF THE AMYLOID PRECURSOR PROTEIN

The transmembrane precursor APP is subjected to proteolytic processing at several sites by a group of proteases collectively called secretases (Figure 1B). Two major pathways, an amyloidogenic and a non-amyloidogenic one, are known, both of which initially involve the shedding of a large ectodomain fragment (sAPP for soluble APP) due to proteolysis close to the transmembrane domain. The amyloidogenic pathway produces the 4 kDa Aβ peptide which is involved in the pathology of AD. Following the processing of the ectodomain, the final cleavage also releases a small fragment with nuclear signaling capabilities. The secretases, referred to as α-, β- and γ-secretases, and their biological implications are discussed below in more detail.

The α-Secretases

During the non-amyloidogenic processing (Figure 1B), APP is cleaved by α-secretases, resulting in the release of the ectodomain fragment known as sAPPα. This processing takes

place within the sequence of Aβ, thus preventing its formation. Besides sAPPα, a C-terminal, membrane associated fragment of 83 amino acids (CTFα, C83) is generated. The sAPPα is released to the extracellular medium and has been shown to have neuroprotective properties (10-12). The α-secretases mainly belong to zinc metalloproteases of the ADAM (a disintegrin and metalloprotease domain) family (13). Especially ADAM-9, -10 and -17 have been shown to cleave APP at Lys16 and Leu17 of Aβ (Lys687 and Leu688 in the numbering of APP_{770}) (14-23). However, the members of the ADAM protein family are not the only proteases shown to process APP. The aspartyl protease BACE2 (β-site APP cleaving enzyme 2), a homologue of the β-secretase BACE1 (see below), also exhibits α-secretase activity but shows a slightly different site specificity in that it cleaves APP between residues Phe19 and Phe20 of Aβ (24).

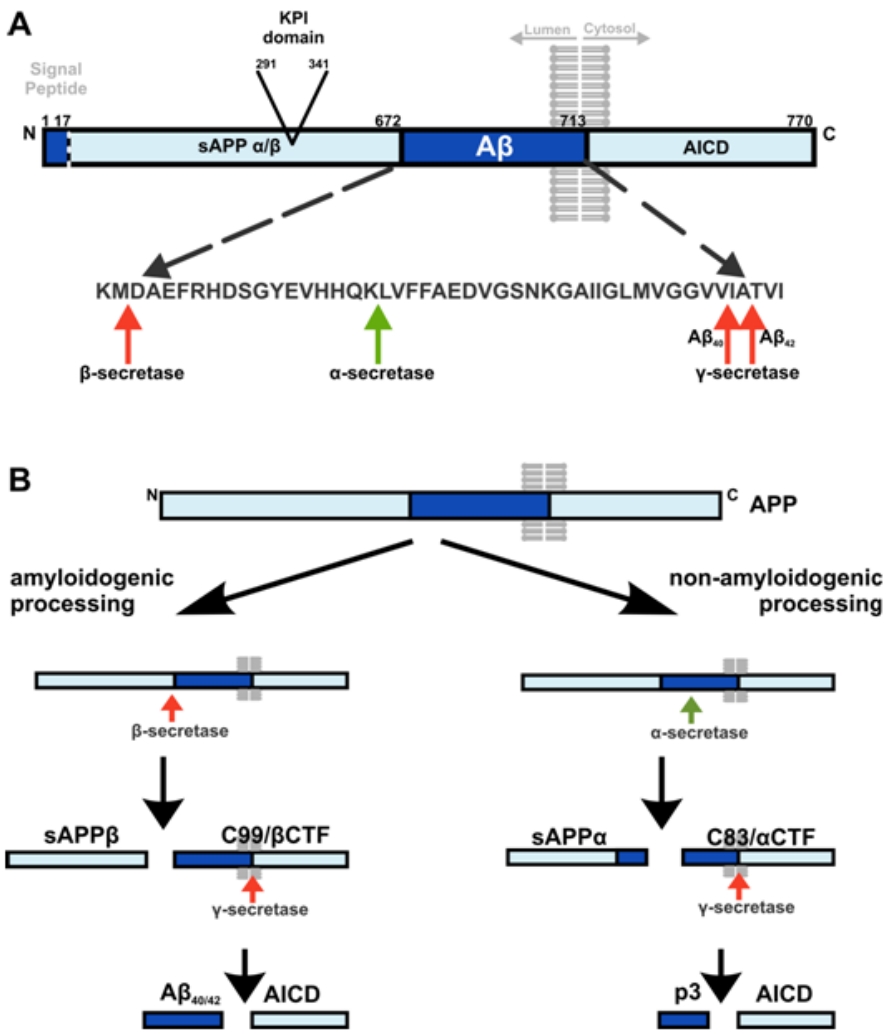

Figure 1. The Alzheimer Amyloid Precursor protein and its processing. APP is a type 1 transmembrane protein which can be proteolytically processed, giving rise to various fragments with biological significance. See text for details.

With the exception of the aspartyl protease BACE2, the α-secretases are members of the ADAM zinc metalloprotease family (13, 25, 26) which show a characteristic conserved domain structure. The N-terminal pro-domain is removed during transport through the Golgi apparatus by pro-protein convertases (27, 28). In addition, the ADAMs contain a metalloprotease domain, disintegrin domain, EGF domain, transmembrane domain and a C-terminal cytoplasmic tail. While ADAMs have been implicated to participate in cell-cell adhesion via their disintegrin domain (29), they are also capable of hydrolysis of protein substrates with their catalytic metalloprotease domain with a zinc binding motif (HEXGHXXGXXHD) (30). In addition to APP, ADAM-9, -10 and -17 are capable of shedding several other type I transmembrane proteins. Remarkably, they predominantly cleave proteins 12 amino acids adjacent to the transmembrane domain (31). In neurons, ADAM-9 and -17 appear to play a minor role as α-secretases (32, 33), whereas ADAM-10 exhibits a physiologically relevant function (34).

Since the initial shedding step of the APP ectodomain is crucial for the decision between the non-amyloidogenic vs. Aβ producing pathway, enhancing the α-secretase activity would direct a major fraction of APP to the non-amyloidogenic route and prevent Aβ formation. This has made α-secretases attractive candidates for strategies aiming at reducing amyloidogenic processing by pushing the α-secretase activity. Agonists such as acetylcholine, carbachol and muscarine that activate muscarinic receptors mediate signaling events leading towards the activation of protein kinase C (PKC), Src tyrosine kinases or mitogen-activated protein kinases (MAPK), which in turn can increase sAPPα production (14, 35). Furthermore, an increased α-secretase activity has been demonstrated as a result of phorbol ester stimulation (13, 36). Recently, Kojro *et al.* provided evidence that the pituitary adenylate cyclase activating polypeptide (PACAP) stimulates α-secretase activity and thus also the non-amyloidogenic processing of APP (37).

The β-Secretase

During the amyloidogenic processing of APP (Figure 1B), the initial shedding of the APP ectodomain sAPPβ, which is 16 amino acid residues shorter than sAPPα, is accomplished by the β-secretase BACE1, also known as memapsin-2 (membrane-anchored aspartic protease of the pepsin family) or Asp-2 (aspartyl protease 2) (38-41). BACE1 is a single-pass, type I aspartic protease (D(S/T)G) (41) that processes APP at residues Met671 and Asp672 (for a review, please see 42, 43), thus liberating sAPPβ and generating a C-terminal fragment of 99 residues, called C99 (38-40, 42-44). The C99 is then processed by the γ-secretase complex (see below), resulting in the release of Aβ and an intracellular domain called AICD. Not only APP but also other proteins including APLP1 and APLP2 can be cleaved by BACE1, consistent with their high homology to APP (45).

Processing by BACE1 can be considered as the rate-limiting step in the Aβ generating cascade, since BACE1 represents the first catalytically active enzyme of the pathway (46). BACE1 contains two active site motifs which reside within the N-terminus on the extracellular side of the membrane and are essential for the proteolytic activity (38, 47). BACE1 exhibits proteolytic acitivity at acidic pH 4.5, suggesting a localization in acidic compartments, such as endosomes along the secretory pathway (40).

The highest expression level of BACE1 is found in the brain, especially in neurons originating from AD brains (38, 40, 48-50). Subcellularly, BACE1 localizes to the plasma membrane from where it gets internalized into early endosomes and/or recycling endosomes (40, 51-53). BACE1 is synthesized as a pro-protein in the ER and then maturated during Golgi transit (47, 54), but removal of the pro-domain is not essential for the proteolytic activity (47, 51). Multiple post-translational modifications of BACE1 include N-glycosylation (55), formation of disulphide bonds (55), dimerization (56), phosphorylation on Ser498 (53), palmitoylation (54) and reversible acetylation of lysine residues (57). In particular, the phosphorylation of Ser498 and a dileucine motif in the cytoplasmic tail are essential for the correct targeting and trafficking of BACE1 (53, 58). Palmitoylation of cysteine residues is essential for anchoring BACE1 to the cell membrane, and especially to lipid raft microdomains (59, 60). In addition, shedding of BACE1 by an as yet unknown sheddase leads to the formation of a soluble BACE1 ectodomain which still exhibits proteolytic activity towards APP (54).

Due to its key role as a rate limiting enzyme during Aβ production, BACE1 appears to be an attractive target for AD therapy strategies. Thus, several studies have been focusing on the modulation of β-secretase activity by means of cholesterol depletion with statins, which might influence BACE1 stability within lipid rafts (reviewed in 42). Others were investigating the role of lipids (61) and glycans or even the activity of other proteins, such as members of the reticulon family (RTN3 and RTN4B/Nogo-B) or the prion protein (PrP). RTN3 and RTN4B were shown to be negative modulators of BACE1 activity which act by directly binding to the BACE1 C-terminus and thereby preventing the access of BACE1 to its substrate APP (62, 63). Furthermore, overexpression of the cellular form of PrP has been reported to influence the β-secretase cleavage of APP and thus to reduce Aβ levels (64). However, care has to be taken with such strategies since BACE1 may have important physiological functions which so far have not been completely characterized. For example, in *BACE1*$^{-/-}$ mice, the lack of Aβ and BACE1 paradoxically causes memory impairments. Furthermore, a role for the β-secretase as a stress-response protein has been proposed (for a review, see 65).

The γ-Secretase Complex

The final and common step of both APP processing pathways is the processing of the C83 or C99 fragment by the membrane-embedded γ-secretase complex. This complex is composed of four integral membrane proteins: presenilins (PSEN-1 or PSEN2) (66-68), nicastrin, anterior pharynx-1 (Aph-1) and presenilin-enhancer 2 (Pen-2) (69). In addition to the APP intracellular domain, the γ-secretase processing generates a short 3 kDa peptide (p3) during the non-amyloidogenic pathway (70-72) and the 4 kDa Aβ peptide during the amyloidogenic pathway (73). The γ-secretase processing of APP takes place within the membrane bilayer, and is thus referred to as regulated intramembrane proteolysis (RIP). The γ-secretase cleavage site may slightly vary within APP, and thus several species of Aβ are generated which are of different length, e.g. $A\beta_{40}$ and $A\beta_{42}$ (72, 74, 75). These Aβ species exhibit different tendencies for aggregation, with $A\beta_{42}$ being highly prone to aggregation and thus being especially disease relevant. Under non-pathological conditions about 90% of the Aβ produced is $A\beta_{40}$, whereas during familial AD, the ratio shifts in favor of $A\beta_{42}$ (74, 76, 77). Mutations of PSEN, which serves as the catalytic subunit for the γ-site cleavage, are

often linked to early onset familial AD (66). Especially mutations in PSEN1 and PSEN2 have been shown to increase the levels of $A\beta_{42}$ in familial AD (78).

Presenilins are integral membrane proteins of the novel GxGD type of aspartyl proteases (68, 79-82), and they span the membrane several times. Recently, a model proposed even nine transmembrane domains for PSEN (83). A previous study showed that during the γ-secretase complex formation, PSEN is endoproteolytically cleaved into an N-terminal fragment (NTF) and a C-terminal fragment (CTF) (84). During the stepwise assembly of the γ-secretase complex, the presenilins are synthesized as holoproteins which are unstable and prone to degradation unless PSEN associates with a pre-formed complex of Aph-1 and nicastrin (85, 86). This pre-formed complex is thought to stabilize and integrate the presenilin holoprotein into the forming γ-secretase complex (87-89). Upon association of Pen-2 with this trimeric complex, endoproteolysis of the presenilin holoprotein between transmembrane domains 6 and 7 to CTF and NTF generates the proteolytically active γ-secretase. Thus, Pen-2 appears to be essential for the generation of the presenilin CTF/NTF heterodimers and the complex formation (81, 90). Nicastrin contributes to the initial recognition of γ-secretase substrates and has therefore been referred to as the "γ-secretase substrate receptor" (91).

The ratio for the γ-secretase components is 1:1:1:1 for PSEN : Pen-2 : nicastrin : Aph-1 (92). Remarkably, there are two different presenilins and three Aph-1 proteins, which all have been proven to be functionally active in the γ-secretase complex. Thus, one can expect that about four to six distinct combinations of γ-secretase complexes might exist (93-95).

CELLULAR FUNCTIONS OF APP

The research on APP during the first years after its discovery primarily focused on the Aβ peptide and its involvement in the progression of AD, while the more physiological functions of APP were neglected for a long time. However, APP evidently does not exist solely to produce Aβ and to induce AD. Hence, during the last years, the elucidation of the physiological functions of APP became a main focus of APP research. Meanwhile, APP and its products are accepted to play important physiological roles, and a variety of functions have been proposed which are briefly summarized below.

Cell Survival and Growth

Of the proposed physiological functions of APP and its products, the most accepted one is the role for sAPPα in promoting neuronal survival. Pretreatment of rat hippocampal and septal neurons or human cortical neurons with sAPPα was protective against hypoglycemic damage and glutamate neurotoxicity (96). Hypoglycemia is normally accompanied by a rise in intracellular calcium, which mediates neuronal damage. Treatment of neurons with sAPPα led to a reduction of the baseline intracellular calcium level and prevented its hypoglycemia-induced rise (96).

APP was proposed to be important for neuronal viability, axonogenesis, and dendrite branching. However, this issue remains somewhat equivocal. Although hippocampal neurons from $APP^{-/-}$ mice showed a reduced viability and neurite outgrowth (97), no difference in

viability between primary neurons from wildtype and APP/APLP2 double knockout mice was observed by Heber *et al.* (98). Similarly, in another study, no differences in glutamate-induced apoptosis were observed between neurons from $APP^{-/-}$ or wildtype mice (99).

The first growth-promoting effect of APP was reported 20 years ago. An antisense-mediated suppression of APP expression in fibroblasts resulted in a reduced growth rate. However, when APP or conditioned growth medium from APP overexpressing cells was added, the fibroblast growth rate was restored (100). The minimal sequence requirement for the growth promoting activity of APP was mapped to the five amino acid sequence $_{328}$RERMS$_{332}$ within APP_{695} (101). Apparently, APP does not modulate the growth of neuronal cells only. The ectodomain fragment sAPPα has been shown to function as a growth factor for thyroid epithelial cells (102, 103). A knockdown of APP in SW837 colon carcinoma cells resulted in a diminished tumor formation in nude mice and reduced cell proliferation which could be rescued by conditioned medium from wildtype cells or by purified sAPP. In addition, epidermal growth factor (EGF) -induced phosphorylation of extracellularly regulated kinases Erk-1 and -2 was reduced and delayed in APP knockdown cells (104). Interestingly, many cancer cells express APP (104), but the possible involvement of APP in carcinogenesis has so far not been conclusively demonstrated.

Neuronal Plasticity and Memory

Neuronal loss and axonal pruning occur after brain injury but are also normal processes in the developing brain that serve to correct for e.g. inappropriate axonal branches. A recent study showed that upon trophic factor deprivation, APP is predominantly cleaved by the ß-secretase. The released sAPPβ is further cleaved by an unknown mechanism to release the approximately 35 kDa N-terminal part (N-APP) of APP which binds to death receptor 6 (DR6) and triggers degeneration of cell bodies via caspase 3 and of axons via caspase 6 (105). Abnormal accumulation of N-APP due to high β-secretase-mediated cleavage may trigger neurodegeneration and hence facilitate the development AD (106).

APP breakdown products also influence the processes of memory formation. In mice, intracerebroventricular injection of sAPP resulted in memory-enhancing effects for both short- and long-term memory (12, 107). Most APP overexpressing mice display memory and learning deficits, and normally, there is a correlation between the amount of amyloid deposition in the brain and the extent of the memory deficit (for a review, please see 108). Memory deficits in APP transgenic mice were successfully reversed upon Aβ-vaccination (109), a method which has also raised high hopes as a potential therapy for AD.

Metal Homeostasis

One hallmark of AD is an abnormal brain metal homeostasis characterized by an accumulation of copper, iron and zinc (for a more detailed discussion, please refer to 110). APP knockout mice as well as APP transgenic mice show disturbances in their brain metal levels that are most pronounced for copper (110, 111). APP contains metal binding sites for copper and zinc, pointing to a role in brain metal homeostasis. Aβ reduces Cu(II) to Cu(I) and Fe(III) to Fe(II) and, thereby, provokes the generation of ROS, i.e., H_2O_2 and hydroxyl

radicals. Hence, a high concentration of Aβ may directly contribute to a redox shift in the brain of AD patients (112). Furthermore, the aggregation of Aβ is mediated by interaction with copper ions (113).

Cell Adhesion

APP was proposed to function as a cell adhesion molecule and/or substrate adhesion molecule, because it binds to extracellular matrix components, such as heparin (114), collagen type I and, though less efficiently, to collagen type IV in a saturable manner (115). APP binding to collagen is inhibited by heparin, suggesting the existence of overlapping binding sites for heparin and APP on collagen (115). Heparin binding in turn is modulated by zinc(II) in an allosteric manner (114).

All three APP family members are able to form homo- and heterodimers in vivo, which promotes calcium-independent intercellular adhesion. Embryonic fibroblasts (MEFs) derived from APP/APLP2 double knockout or APLP2$^{-/-}$ but not APP$^{-/-}$ mice display a reduced adhesion capability. APLP2 was confirmed to be required and sufficient for adhesion, because re-transfection of APLP2 into the double knockout MEFs restored their adhesion properties (116).

Regulation of Gene Expression

The capability of APP to promote transcriptional activation has been a topic of two recent reviews (117, 118) and will only briefly be discussed here. After the γ-cleavage, the AICD forms a transcriptionally active ternary complex with the adaptor protein Fe65 (see below) and histone acetyltransferase Tip60 (119). It is now widely accepted that AICD itself has no active role in transcription but rather acts by activating Fe65. This activation is a prerequisite for Fe65 nuclear translocation and takes place at the membrane. After the γ-cleavage, the complex of activated Fe65 together with AICD is released and enters the nucleus (120). Fe65/APP/Tip60-dependent transcription is further modulated by other factors such as 14-3-3γ which directly binds to AICD and Fe65 and thereby enhances their association (121). Despite being transcriptional activators for some genes, AICD and Fe65 were also shown to negatively regulate transcription, i.e. they bind to the EGF receptor promoter (122) and the lipoprotein receptor like protein 1 promoter (123), suppressing their transcription. Several putative AICD target genes have recently been identified (117, 124), but need to be further validated.

ANIMAL MODELS FOR THE APP FAMILY PROTEINS

Knock-Out Animals

Biological clues to the elusive function of APP and APLPs have been obtained through studies using knock-out strategies in animal models such as worms, fruit flies and mice. The nematode *C. elegans* has only one APP-related gene, and loss of this APP orthologue *apl-1* leads to morphological defects and lethality in the larval stadium. Neuronal expression of the

apl-1 ectodomain is sufficient to rescue the lethality, suggesting that the biological function of apl-1 in the worm can be attributed to the extracellular part of the protein (125). Fruit flies (*Drosophila melanogaster*) with a deletion of their single APP-like gene (*appl*) are viable, fertile and morphologically normal, but tend to have a phototaxis defect which can be rescued by re-expressing drosophila APPL or human APP_{695} (126). Mutation of *appl* affects axonal transport by leading to axonal accumulations of transported components (127).

The generation of APP knockout mice was anxiously awaited and was almost simultaneously accomplished by two different groups (128, 129) which applied different cloning strategies. While Müller *et al.* (128) disrupted the murine APP gene by inserting a neomycin cassette into exon 2, leading to the production of a shortened transcript and a truncated protein, Zheng *et al.* (129) deleted the APP promoter and exon 1, resulting in a complete lack of APP. Surprisingly, the phenotype of both mouse lines was unexpectedly mild, suggesting that APP actually is dispensable. $APP^{-/-}$ mice are viable and fertile but have a slightly reduced body weight and show certain behavioral deficits, i.e. impaired spatial learning in the Morris swimming test (128) and reduced locomotor activity (129). In addition, $APP^{-/-}$ mice have decreased forelimb grip strength, indicating compromised muscular functions. The brains of $APP^{-/-}$ mice appeared normal except for the presence of reactive gliosis of unknown etiology (129). These deficits, namely the reduced body weight, grip strength, spatial learning and locomotor activity, could be rescued by knock-in of the sAPPα ectodomain, indicating that the C-terminal part of APP is dispensable for the physiological functions of APP (130). Later studies revealed that $APP^{-/-}$ mice are more susceptible to kainic acid induced seizures. Upon kainic acid injection, seizures initiated earlier, were more severe and resulted in a higher mortality rate in knockout than in wildtype animals (99). $APP^{-/-}$ mice show elevated copper levels in the brain and the liver, whereas zinc and iron concentrations remain unchanged. Normally, brain copper levels are strictly regulated due to copper's high capacity to generate free radicals, and hence, a disturbance of copper levels could contribute to neuronal damage (131). Cholesterol homeostasis also seems to be disturbed in $APP^{-/-}$ mice because brain ApoE levels were found to be decreased by 50% due to an increased expression and binding capacity of the ApoE receptor, LRP1. These changes were accompanied by an increase in the brain cholesterol (123).

The reason for the mild phenotype of APP knockout mice in contrast to the lethal phenotype in *C. elegans* is due to partial compensation by the other APP family members, indicating a functional redundancy, which considerably complicates the revelation of APP *in vivo* functions. For the compensation to take place, the basal expression level of APP homologs seems to be sufficient, since $APP^{-/-}$ mice did not show differences in APLP1 or -2 expression (129). The deletion of either APLP1 or -2 also resulted in mild phenotypes. $APLP1^{-/-}$ mice show a somatic growth deficit as the only abnormality (98), while $APLP2^{-/-}$ mice have no apparent phenotype at all (132), except for the increased copper levels in the brain and the liver (131).

Since single knock-outs of the APP family failed to reveal the biological significance of these proteins, all combinations of double mutants were generated (133). Most APP/APLP2 and APLP1/APLP2 double knockout mice die within one week after birth. The reason for the lethality is not fully understood because the mice lack obvious histopathological abnormalities. Only recently it was reported that $APP/APLP2^{-/-}$ mice show an impaired development of neuromuscular synapses on day P0, characterized by excessive nerve terminal sprouting, a reduced number of synaptic vesicles at presynaptic sites, a defective

neurotransmitter release and high incidence of synaptic failure. Most likely, the lack of sAPPα with its neurotrophic and synaptogenic properties is responsible for the observed abnormalities (134). Even more strikingly, one day old APP/APLP2 double knockout mice have drastically reduced plasma glucose levels, but simultaneously suffer from hyperinsulinemia due to insulin hypersecretion. Plasma corticosterone levels are also elevated, indicating that APP and APLP2 modulate glucose and insulin homeostasis and hence play a role in energy regulation. The cause of perinatal death of those animals is evidently the lethal hypoglycemia (135). Approximately 20% of the APP/APLP2 double knockout mice survive into adulthood and show a reduction in the body weight, poor mating and marked behavioral abnormalities, i.e. difficulty in righting, ataxia and spinning behavior, but no gliosis or organ abnormalities (132).

In contrast, a double knockout of APP and APLP1 is comparable to $APP^{-/-}$ mice. Deletion of one APLP2 allele in APP/APLP1 double knockout mice resulted in postnatal lethality, indicating that one APLP2 allele is not sufficient for survival in the absence of other APP family members, corroborating a key physiological role for APLP2 (98). Overall, the results with the double knockout animals point towards a role for APP family members in early postnatal development. The loss of one family member is to a large extent compensated by the others, whereas a double knockout involving APLP2 is perinatally lethal. A combined deletion of all three APP family members is lethal, partly due to cranial abnormalities, i.e. cortical malformations and partial loss of cortical Cajal Retzius cells, suggesting a crucial role for APP family members in the brain development (136).

Transgenic Mouse Models

Several transgenic mouse models that allow the study of AD have been established (137). All of them show hallmarks of human AD, i.e. accumulation of Aβ, eventually amyloid plaque formation in the brain and symptoms of neurodegeneration. The extent and onset of plaque formation and neurological symptoms is dependent on the level of overexpression, on the promoter of the expression construct and on the presence of mutations within the overexpressed APP. So far, approximately 20 APP missense mutations have been identified, some of which are associated with early onset of familial Alzheimer's disease (FAD) in humans. These mutations are mostly located near the Aβ region and lead to a higher production of the more fibrillogenic $Aβ_{42}$ (118).

In 1995, so-called "PD-APP mice" overexpressing human APP carrying a FAD-associated mutation (V717F) driven by the PDGF-ß promoter were generated (138). Those mice have an approximately 10-fold overexpression of APP and show plaque formation, gliosis, loss of synaptic density (138) and age-dependent spatial memory deficits (139) starting at 6-9 months of age. Similarly, "Tg2576 mice" overexpressing human APP carrying a double Swedish mutation (i.e. K670N, M671L) showed plaque development as well as learning and memory defects in the age of 9-10 months (140). "APP23 mice" overexpressing human APP with Swedish mutations under the control of the Thy-1 promoter at a level 7-fold above that of endogenous APP develop amyloid plaques already at 6 months of age (141).

Not only APP transgenic mice develop hallmarks of AD. Also mutations in presenilin 1 and 2, the catalytic components of the γ-secretase complex, contribute to early-onset familial AD. While overexpression of wildtype presenilins alone did not result in increased $Aβ_{42}$ formation, transgenic mice overexpressing mutated variants of presenilins show an increase in

the brain $A\beta_{42}$ production (142). Double transgenic mice overexpressing mutant presenilin 1 and APP even showed an accelerated plaque deposition and an increased ratio of $A\beta_{42}/A\beta_{40}$, as compared to single transgenic mice (143, 144).

To form Aβ, APP needs to be cleaved by β- and γ-secretases. Hence, also BACE1 knockout and overexpressing mice were in the focus of interest. Both of them are apparently normal and do not display obvious abnormalities (145, 146), suggesting that overexpression of BACE1 is not sufficient to produce β-amyloid plaques. Knockout of BACE1 in APP Tg2576 transgenic mice prevented plaque formation (147), while BACE1/APP double transgenic mice showed an accelerated plaque deposition (146).

CELLULAR LOCALIZATION AND TRAFFICKING OF APP

After its synthesis in the ER, APP is directed to the secretory pathway and transported through Golgi/TGN towards the plasma membrane. During its transit through these compartments, APP is subjected to several posttranslational modifications, such as N- and O-glycosylation, phosphorylation and tyrosine sulfation. In many cell types, however, less than 10% of APP is detected at the cell surface in steady state, whereas the major pool of APP is localized at the Golgi/TGN regions. In neuronal cells, APP is also detected in axons and synapses.

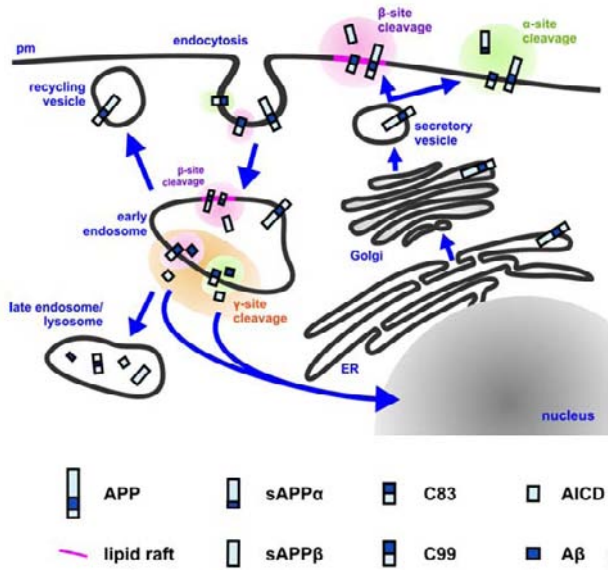

Figure 2. Cellular compartments involved in APP processing. After its synthesis in the ER, APP is transported through the Golgi apparatus. Depending on the compartment APP is transported to, it is subjected to processing by the secretases. Whereas α-secretases are likely to cleave the cell surface associated APP, β- and γ-secretase mediated processing rather takes place intracellularly, mainly in endosomes.

Cellular localization of APP is a major factor in determining the accessibility of APP as a substrate for the secretases. Whereas cell surface resident APP is likely to be processed by the α-secretases, APP in the intracellular compartments (TGN and endosomes) is rather subjected

to processing by β- and γ-secretase (Figure 2). Thus, endocytosis of APP from the plasma membrane to endosomes should enhance the amyloidogenic processing of APP.

The cellular targeting of membrane proteins is generally regulated by means of binding of adaptor proteins in the sorting motifs residing in the cytoplasmic domains of the cargo proteins. Two major sorting signals have been identified in the cytoplasmic tail of APP which is relatively short, comprising only 47 amino acids. The membrane proximal Y^{653}TSI motif has been shown to bind to PAT-1 (protein interacting with APP tail 1) and the tetrameric adaptor complex AP-1B (148, 149). Several binding partners have been described for the more distal Y^{682}ENPTY^{687} sorting signal. The interaction partners of APP tail and their putative role in the trafficking of APP are summarized in Figure 3 and discussed in detail below.

The YTSI Sorting Motif: Polarized Targeting in Epithelial Cells

Early findings demonstrated that in polarized Madine-Darby canine kidney (MDCK) cells, APP was almost exclusively transported to the basolateral membrane, and sAPP was secreted predominantly in the basal medium. The basolateral transport of APP was shown to depend on Y^{653}, the mutation of which abolished the polarized sorting of APP (150). However, secretion of sAPP still took place in a polarized fashion, most likely due to the polarized distribution of the α-secretase in the basolateral compartment (151).

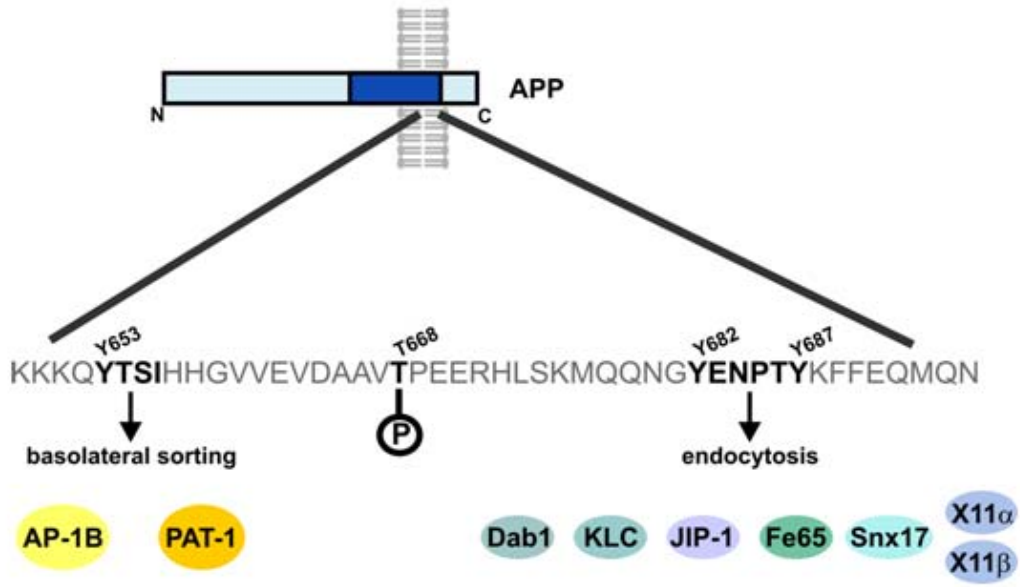

Figure3. Binding partners of the sorting motifs in APP intracellular tail. Two major sorting signals are responsible for the sorting of APP in the cells. The membrane proximal YTSI motif mediates the basolateral targeting of APP in polarized epithelial cells, whereas the more distal YENPTY signal is responsible for e.g. endocytosis. For both motifs, specific binding partners which are involved in APP targeting have been described. Please see text for details.

The heterotetrameric adaptor complexes have been shown to be involved in many sorting events of transmembrane proteins in the cells (for a review, see 152). So far, 4 different adaptor complexes have been identified, termed AP-1, AP-2, AP-3 and AP-4. AP-2 is mainly involved in endocytosis through clathrin coated pits, whereas AP-3 mediates the transport of proteins into lysosomes and lysosome-related organelles such as melanosomes and platelet α-granules. The AP-1 complex is involved e.g. in the sorting of cargo proteins between TGN and endosomes. The role of AP-4 in cellular trafficking still remains somewhat obscure, although it has been suggested to function in polarized sorting (153). However, basolateral targeting of some transmembrane proteins, including low density lipoprotein receptor (LDLR), was shown to depend on an epithelia-specific form of the adaptor complex AP-1, termed AP-1B (154-156). The difference between the epithelial and ubiquitous forms of AP-1 is the incorporation of two different isoforms of the μ subunit, with AP-1 exhibiting the μ1A and AP-1B the epithelial μ1B subunit, which have different cargo binding specificities.

The μ1B subunit of AP-1B binds to sequences that conform to the consensus Yxxϕ (with ϕ representing a bulky hydrophobic residue). We were recently able to show that μ1B binds to the Y^{653}TSI sorting signal in the cytoplasmic tail of APP and mediates its basolateral targeting in epithelial cells (148). Porcine kidney LLC-PK1 cells, which do not express μ1B, are not capable of sorting basolateral cargo into the correct domain. In these cells, APP is randomly distributed in apical and basal membranes, but ectopic expression of μ1B is sufficient to rescue the basolateral localization of APP.

In addition to AP-1B (148), so far only one other protein, PAT-1, has been shown to bind to the YTSI sorting signal in APP (149). Since PAT-1 shows homology to kinesin light chain (KLC), it was speculated that it might be involved in transport of APP-containing vesicles along microtubules. However, in many cell lines, PAT-1 was later shown to be localized mainly in the nucleus, thus making a function in cytosolic transport processes unlikely (157). On the other hand, more recent findings have suggested that PAT-1 is able to induce a cell death signal by means of increasing the amount of APP and APLP2 at the cell surface (158). In addition, PAT-1a, which shows 99% homology to PAT-1, interacts and colocalizes with APP, APLP1 and APLP2 in TGN vesicles or endosomes and increases the amyloidogenic processing of APP, suggesting that this protein might indeed play a role in the regulation of APP trafficking and processing (159).

Binding Partners of the YENPTY Motif

Several proteins which influence the trafficking and/or processing of APP have been shown to bind to the distal YENPTY motif which conforms to the consensus NPxY type of sorting signal. NPxY motifs are typically found in many transmembrane proteins and have been shown to e.g. mediate their endocytosis. The binding partners of APP interacting with the YENPTY sequence include Disabled-1 (Dab1), sorting nexin 17, the members of the Fe65, X11 (also known as Munc-18 interacting proteins or Mints), Shc, and c-Jun N-terminal kinase interacting protein (JIP-1) families (160-163). These proteins contain a phosphotyrosine binding (PTB) domain which has been suggested to mediate the interaction with APP. Phosphorylation of the APP tail has also been shown to occur on several sites and

to play a role in the regulation of trafficking, although it appears that some of the PTB-containing interaction partners do not bind to the phosphorylated tail (see below).

The neuronal isoforms of the X11 family, X11α and X11β, both interact with the APP tail and have been shown to influence APP processing, although the exact molecular mechanism has not yet been clarified. X11 proteins have been suggested to be Arf GTPase dependent vesicle coat proteins, making their role in trafficking processes plausible (164). Interaction of APP with X11 appears to influence synapse formation (165), in line with the function of X11 proteins in e.g. synaptic vesicle exocytosis and neuronal copper homeostasis (166, 167). Very recent findings with the X11-like protein imply that Ser phosphorylation of the N-terminal region outside of the PTB domain of X11L regulates the interaction with APP (168). Several findings have suggested that overexpression of X11 proteins would stabilize APP and exhibit an inhibitory effect on amyloidogenic processing of APP. However, RNA interference studies imply that downregulation of X11 expression also results in reduced Aβ production, thus contradicting the role of X11 in APP processing (169). The most likely explanation for this controversy is that a balanced amount of X11 is a prerequisite for correct APP processing, and thus both overexpression (possibly due to a dominant negative effect) and downregulation of X11 proteins decrease amyloid production. Furthermore, *in vivo* studies using transgenic Alzheimer mouse models or XL11L-deficient mice would suggest that X11 proteins inhibit amyloidogenic APP processing and thus reduce the amyloid load (88, 170, 171).

The PTB/PID domain containing adaptor protein Fe65 has been shown to interact with the YENPTY motif of APP (160, 161, 172). Several findings have suggested that interaction of Fe65 with AICD results in signaling to the nucleus and transcriptional regulation (119, 120, 173). Although Fe65 undoubtedly also affects the processing of APP and β-amyloid production, there is some discrepancy in the literature as to whether Fe65 overexpression promotes or inhibits Aβ production (174-178). Fe65 directly binds to APP, and this binding has been suggested to be inhibited by phosphorylation of APP in Thr668 (174) which appears to induce a conformational change in the cytoplasmic domain of APP (179).

Although several APP interactors bind to the YENPTY motif, at least some of them appear to interact with different subsequences therein. For example, phosphorylation of Thr668 in APP reduced binding to Fe65, whereas interaction with X11 proteins and Dab1 was not affected (174). Furthermore, X11 and Fe65 have been shown to bind to distinct sites of the YENPTY motif (180), although later findings have suggested that Fe65 and X11 might compete for the binding in APP (181). Evidence for a competition was also provided by the findings showing that X11 proteins inhibit the nuclear signaling of the Fe65-APP complex (182). Thus, X11 and Fe65 proteins have turned out to be important regulators of APP metabolism and might even play a role in the AD pathology.

APP has also been shown to interact with KLC (183) and to associate with the cytoplasmic domain of LDLR-like Protein (LRP) (102, 184-187). Kamal *et al.* suggested that axonal transport of APP is mediated by direct interaction with KLC (183). They proposed that APP might function as a kinesin receptor on transport vesicles and the APP processing machinery (BACE1 and γ-secretase) could be packed in these vesicles, thus facilitating the proteolytic processing of APP during its anterograde axonal transport. However, other researchers have been unable to verify these findings, thus questioning the role of APP as a receptor in kinesin-mediated transport (188).

LRP has been shown to modulate several steps in the sorting and proteolytic processing of APP (102, 184). However, recent findings have suggested that its interaction with APP might not be a direct one but rather mediated by the PTB-containing adaptor Fe65 (187, 189). Interestingly, the region necessary for the association of APP with LRP was mapped into a sequence NPTYATL in the LRP tail (102, 187), which also contains a putative NPxY motif that represents a binding site for Fe65 (190). Thus, Fe65 appears to function as a linker between APP and LRP by means of binding to the NPxY motifs in these proteins with its two distinct PTB/PID domains.

Role of YENPTY Motif in the Endocytosis of APP

Endocytosis of APP from the cell surface has been shown to be important for the proteolytic processing of APP by the β-secretase BACE1 in endosomes, which are the major site of action of BACE1. Inhibition of APP endocytosis has been shown to enhance the shedding of APP ectodomain and concomitantly decrease the amyloidogenic processing (191). In some cell types, however, amyloidogenic processing of APP can also take place at the cell surface (192). The major endocytosis signal of APP has been suggested to reside within the GYENPTY sequence (193). Although NPxY motifs are well-known endocytosis signals, it turned out that the dominant endocytosis motif for APP is actually provided by the tetrapeptide YENP, whereas mutation of the Tyr in the NPTY sequence had no effect on APP endocytosis (191).

Early findings have shown that APP is present in clathrin coated vesicles (194, 195), which, however, cannot be considered as a direct proof for clathrin-mediated endocytosis. Uptake of APP from the plasma membrane has been shown to be dependent on dynamin (59, 196), a GTPase that plays a major role in budding of vesicles from the plasma membrane, and expression of a dominant-negative dynamin mutant (197) inhibits APP endocytosis (59, 192). Furthermore, a polymorphism in the dynamin-2 gene has been linked to susceptibility for late-onset AD, and dynamin-2 mRNA was shown to be significantly reduced in AD brains (198, 199).

However, not only clathrin-mediated endocytosis, but also some other endocytosis pathways are dynamin dependent. Only recently, solid proof for clathrin-mediated endocytosis of APP has been provided (200). The authors showed that APP endocytosis is mediated by a specialized clathrin-, AP2 and cholesterol-dependent pathway but is independent of the adaptor protein epsin1 (200). In addition, the lipid-raft protein flotillin-2/reggie-1 was shown to enhance APP endocytosis (198, 199) (see below).

Another protein shown to play a role in APP endocytosis is sorting nexin 17 (Snx17). Snx17 is an adaptor protein that colocalizes with APP in early endosomes and binds to the YxNPxY motif (201). Overexpression of a dominant negative form of Snx17 and its depletion by means of RNA interference both resulted in increased Aβ production and decreased steady state levels of APP. These findings suggest a role for Snx17 in the regulation of APP endocytic/endosomal trafficking and proteolytic processing.

Apolipoprotein E (ApoE) can stimulate APP endocytosis by binding onto the apolipoprotein E receptor 2 (ApoER2) and thus increase the production of Aβ (202). As with LRP and Fe65 binding to APP, the X11 proteins seem to be capable of binding to both ApoER2 and APP, and thus facilitating APP uptake and processing. This might have

important implications for AD, since the ApoE4 form of the ligand has been genetically linked to AD and seems to result in production of higher amounts of Aβ as compared to ApoE2 or ApoE3, likely by stimulating APP endocytosis (202).

Phosphorylation and Sorting of APP

The cytoplasmic tail of APP contains eight potential phosphorylation sites and has been shown to be phosphorylated in several residues. Thr and Ser phosphorylation in Thr654 and Ser655 have been demonstrated in rat brain (203). Both residues reside within the YTSI sorting signal of APP that mediates the basolateral targeting in polarized epithelial cells, but the role of phosphorylation of these residues in the polarized sorting of APP has not been clarified further. Very recent findings suggest that Ser655 phosphorylation increases the secretory trafficking of APP (204). In addition to these two sites, Thr668 has been shown to be phosphorylated by several different kinases *in vitro*, these including glycogen synthase kinase 3β, Jun N-terminal kinase 3, Cdc2 and Cdk5 (205-208). Phosphorylation of APP in Thr668 has been shown to be elevated in the brain of AD patients, and Thr668 phosphorylated APP colocalizes with BACE1 in enlarged endosomes in hippocampal neurons of AD patients (209). Furthermore, the C-terminal fragment generated by the β-secretase is phosphorylated to a higher degree as compared to the α-secretase cleavage product (209). Phosphorylation of Thr668 has also been suggested to enhance the γ-secretase mediated processing of APP, thus presumably promoting the amyloidogenic processing of APP by both β- and γ-secretases (210).

In addition to Thr and Ser phosphorylation, APP is subjected to Tyr phosphorylation in Tyr682, at least *in vitro*, by c-Abl and nerve growth factor receptor TrkA, and phosphorylation of Tyr682 enhances the interaction with the PTB/PI domain adaptor protein Shc (211). Furthermore, phosphorylation of APP in Tyr687 has also been suggested to take place (212). Evidence for the Tyr-phosphorylation of APP *in vivo* has been provided by a study showing that APP is phosphorylated in cells expressing a constitutively active form of Abl kinase (213). Furthermore, c-Abl was recently suggested to modulate the cellular effects of AICD, and a complex containg AICD, c-Abl and the Fe65 adaptor was detected (214). TrkA overexpression has also been shown to result in increased phosphorylation of APP in Tyr682, suggesting that also Tyr phosphorylation plays a role in the regulation of APP metabolism (211).

Disabled-1 (Dab1) is a PTB-PI domain containing adapter protein that plays a role in signal transduction processes (215, 216) and directly binds to APP and APLPs (162, 190, 217). Coexpression of APP family members results in increased serine phosphorylation of Dab1 (162). Similarly to X11 and Fe65 (180), Dab1 also preferentially binds to the nonphosphorylated NPxY motif of APP (162), and phosphorylation has even been shown to inhibit Dab1 binding to APP (217). Thus, in some cases Tyr phosphorylation may negatively regulate the interaction of APP with specific interaction partners and thereby modulate the trafficking and processing of APP.

Sorting of APP in the Golgi and Endosomes

Sortilin-related receptor with A type repeats (SORLA) is a further interaction partner of APP that can influence its cellular sorting and processing. SORLA is a type 1 transmembrane protein that directly interacts with APP, and the cytoplasmic and extracellular domains of both proteins appear to contribute to the interaction (218, 219). The complement-type repeats in SORLA ectodomain mediate the interaction with the carbohydrate-linked domain of APP (218). Overexpression of SORLA results in reduced amyloidogenic processing of APP, whereas mice genetically ablated for SORLA exhibit increased amounts of Aβ in the brain (220, 221). Furthermore, the expression level of SORLA in the brains of AD patients has been shown to be decreased, and SORLA is genetically linked to late-onset AD (222, 223). SORLA can also influence the signaling mediated by APP processing fragments and induce neurogenesis, most likely by means of increasing the secretion of the mitogenic sAPPα (224). Although SORLA has been suggested to localize in both Golgi and endosomes (220, 225), recent findings suggest that SORLA would exhibit its effect on APP processing by regulating the anterograde trafficking of APP from Golgi to plasma membrane (226). However, SORLA does not appear to play a direct role in APP endocytosis (219). The function of SORLA in APP targeting was shown to be dependent on its interaction with two adaptors, GGA and PACS-1, which are involved in sorting of proteins in the Golgi (226). Interestingly, GGA-1 has been previously linked to APP processing although it does not directly interact with APP (227).

Endosomes are intracellular tubular/vesicular compartments that play a key role in protein sorting. Altered endosomal function has been characterized in a wide range of neurodegenerative diseases. In AD, one of the earliest neuropathological features is altered endocytosis in neuronal cells (228-231). Neuronal cells are especially vulnerable for any impairment of endocytosis and/or endosomal function since they extensively depend on endocytosis and endosomal sorting. For example, recycling of synaptic vesicles and neuronal signaling requires endocytosis and involves endosomes. Thus, endosomal dysfunction appears to be highly relevant for the development of AD.

The small GTPase protein Rab5 is involved in endosome function and fusion (232, 233). Interestingly, at very early stages of AD, neurons exhibit Aβ-positive enlarged endosomes which also contain Rab5 (234). High levels of lysosomal hydrolases can be detected in these abnormal endosomes due to their missorting (229), and the levels of cation-dependent mannose-6-phosphate receptor are elevated (235), indicating a failure in lysosomal sorting. Although increased Aβ levels are detected within the endosomes, accumulation of Aβ does not seem to be the cause of endosomal enlargement (236). Molecular mechanisms of the sorting of APP within endosomal structures have not been completely clarified. However, several binding partners of APP, including X11, Fe65 and SORLA, which regulate APP processing may also directly influence its endosomal trafficking.

Association of APP with Membrane Rafts

Cholesterol has now been firmly established as an important factor in the regulation of APP processing. Already a decade ago, it was shown that in AD patient brains, the amount of Aβ correlates with the amount of cholesterol in the serum (237). In cultured cells and AD

animal models, Aβ generation can be influenced by regulating the amount of cellular cholesterol (238, 239). Epidemiological evidence suggest that high blood cholesterol may increase the risk of developing AD later on in life (240), and amyloid plaque formation is enhanced in animals subjected to high cholesterol diet (241, 242). Recently, cholesterol has also been suggested to exhibit a protective effect against amyloid induced membrane damage (243).

During the recent years, the dependency of APP processing on cholesterol has been connected to subcellular structures known as lipid rafts. These membrane microdomains are highly enriched in cholesterol and sphingolipids and have been postulated in regulating processes such as signal transduction and membrane trafficking (244, 245). Rafts are highly variable both in their size (10 nm to micrometers) and consistency, and they have been detected in various cell organelles, e.g. plasma membrane, endosomes and Golgi region. Depletion of cellular cholesterol frequently results in impairment of raft function and has thus been intensively used for studying cellular processes associated with rafts. In addition, rafts tend to be resistant against extraction of membranes with cold nonionic detergents such as Triton X100 (246, 247).

Processing of APP and generation of Aβ has been shown to be dependent on cellular cholesterol, and cholesterol depletion results in reduced Aβ production (59, 239). In fact, amyloidogenic processing of APP has been strongly implicated to take place within rafts, and a fraction of APP is localized within these microdomains (239). In addition to APP, both BACE1 and the active γ-secretase have also been detected in rafts (59, 61, 248-252). Being sensitive to cholesterol depletion, BACE1 is likely to associate with lipid rafts within the plasma membrane and in early endosomes (59, 60, 253, 254). Thus, a concept has emerged according to which APP is subjected to amyloidogenic processing by the raft associated β- and γ-secretases. The non-raft APP in turn becomes processed by the α-secretases such as ADAM10, which are not raft-resident, as evidenced by the fact that ADAM10 was found to be insensitive to cholesterol depletion (253). However, a recent study showed that palmitoylation-deficient BACE1 mutants which should show a reduced raft affinity, were still capable of APP processing, suggesting that β-cleavage can also take place in non-lipid raft microdomains (255). Since endocytosis of APP is a prerequisite for amyloid production in many cell types, it has been postulated that APP meets the β-secretase and becomes processed only after endocytosis in endosomes (59).

Antibody crosslinking or homodimerization of APP have been shown to enhance Aβ production (59, 256), and crosslinking also increased raft association of both APP and BACE1 (59). In line with this, a high molecular weight complex with enhanced BACE1 activity has been suggested to be localized in rafts (250). Recruitment of BACE1 into rafts is also facilitated by its multiple palmitoylation, a modification that increases the affinity of membrane proteins towards rafts (54). In addition, glycophosphatidyl-inositol (GPI) anchored proteins have been suggested to play a role in the recruitment of BACE1 into rafts (60).

Although APP is partly associated with rafts, how the localization of APP into rafts is regulated has not been clarified in detail. Very recent findings have suggested that X11 proteins are associated with the non-raft pool of APP, and in the absence of X11 and X11L, APP is increasingly recruited into rafts and processed by the β-secretase (257). Interestingly, GPI-anchored prion protein (PrP), which is localized in rafts, can also regulate the generation of Aβ, and prion protein was shown to inhibit β-secretases in a manner that was dependent on its interaction with glycosaminoglycans (64). Expression of mutant variants of PrP that cause

familial Creutzfeldt-Jakob disease also result in increased Aβ production (64), although some findings would suggest that PrP rather promotes Aβ aggregation than enhances its synthesis (258). In animal models, infection of an APP transgenic mouse with prions results in rapid development of scrapie-like disease and accumulation of Aβ (259). Recently, impaired APP processing caused by prions was linked to phosphorylation by Src kinases and subsequent degradation of the APP interactor Dab1 (260). Thus, a plethora of evidence supports an important role for PrP in AD and in the regulation of APP metabolism.

Another interesting family of raft associated proteins that has been connected to AD is the reggie/flotillin proteins. Flotillin-1/reggie-2 and flotillin-2/reggie-1 were originally discovered as putative neuronal regeneration proteins in the goldfish (261), but have meanwhile been shown to be highly ubiquitous. Upon lesion of the optic nerve, reggies were highly expressed in the regenerating axons of the retinal ganglion cells (261). In parallel, another group detected flotillins as novel proteins associated with membrane rafts (262). Despite their high degree of evolutionary conservation, the function of these proteins has remained somewhat enigmatic. Recent findings have linked flotillins with various cellular processes including signal transduction and endocytosis (for review, see 263, 264).

Various findings implicate flotillins in AD pathology and APP trafficking. In AD, flotillins have been shown to accumulate in the cortex upon progression of AD (265), and flotillin-1 was found to be enriched in the lysosomes of tangle-bearing neurons in AD brains (266). Intracellular accumulation of Aβ is a feature associated with various transgenic AD animal models (267-270) but also found in Alzheimer brains (271, 272). Flotillin-1 was shown to localize in endosomes together with the intracellular Aβ, but also within extracellular amyloid plaques in AD brain sections (273). However, the functional significance of these findings is not clear.

Since flotillin-1 interacts directly with both APP and BACE1 (274, 275), it is highly plausible that it directly affects either the trafficking or processing of APP. As a raft associated protein, it might even play a role in the recruitment of APP or BACE1 into rafts. Flotillin-1 has been suggested to define a novel clathrin independent, raft mediated endocytosis route which is dynamin dependent (276). However, flotillin-1 knockdown appears to affect neither the endocytosis nor the processing of APP, whereas downregulation of flotillin-2 impairs endocytosis, with concomitant decrease in Aβ production (200). Knockdown of flotillin-2 also results in severely reduced expression of flotillin-1 in animal cells (200, 277) and in flotillin-2 knockout mice (our unpublished findings), and flotillins strongly tend to form hetero-oligomers (277, 278). Nevertheless, the results of Schneider *et al.* would implicate that the observed effect on APP processing is purely caused by flotillin-2 (200). So far, however, only flotillin-1 has been shown to directly interact with APP (274) and our unpublished results would indicate that the interaction between APP and flotillin-2, if any, is much weaker than that between APP and flotillin-1.

Since endocytosis of APP takes place by means of a clathrin dependent pathway (200), whereas flotillins rather utilize a raft mediated route (276), the question arose how flotillin-2 affects the endocytosis of APP. Interestingly, expression of dominant negative (DN) dynamin-2 in neuroblastoma cells impairs APP endocytosis but still enhances Aβ production. This might be due to the fact that DN dynamin-2 expression appears to result in increased accumulation of APP in flotillin rich rafts at the plasma membrane (199) and perhaps mistargeting of β-secretase into these rafts. Frequently, clustering of proteins into rafts takes place during signaling and trafficking processes. Flotillin-2 was suggested to be essential for

the clustering of APP into microdomains which then facilitate APP endocytosis (200). Clustering of APP with antibodies in flotillin-2 depleted cells resulted in normal endocytosis of APP, further supporting this hypothesis (200). Thus, although APP is endocytosed by means of clathrin coated pits, flotillin-driven clustering at the plasma membrane appears to be a prerequisite for a productive endocytosis.

CONCLUSION

Intracellular trafficking and cellular compartmentalization of APP has been shown to be a major factor in determining which secretase gains access to APP, which in turn is the key event during decision between amyloidogenesis and non-amyloidogenic processing. Depending on the cellular compartment that APP resides in, it becomes accessible to different binding partners, which can influence its trafficking and thus also processing. Therefore, these binding partners of APP, both the known ones and the as yet unidentified ones might also turn out to be highly important for understanding the cellular mechanisms that regulate APP processing and result in manifestation of AD.

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Chapter 2

THE NA/K-ATPASE ENDOCYTOSIS AND SIGNALING

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ABSTRACT

The Na/K-ATPase was discovered as an energy transducing ion pump. A major difference between the Na/K-ATPase and other P-type ATPases is its ability to bind a group of chemicals called cardiotonic steroids (CTS). Endogenous CTS have been identified as a new class of endogenous hormones, functioning as important regulators of renal Na^+ excretion and blood pressure. Na/K-ATPase is not only an ion pump, but also an important receptor that can transduce ligand-like effect of CTS on intracellular protein kinases. Significantly, the CTS-provoked kinase cascades are capable of inducing endocytosis of the apical NHE3 (Na/H exchanger isoform 3) and basolateral Na/K-ATPase in renal proximal tubular cells. Functionally, this CTS-induced coordinately regulation of Na/K-ATPase and NHE3 leads to the inhibition of sodium reabsorption in renal proximal tubules under physiological conditions, such as high salt diet. A defect in this regulation would reduce the ability of renal proximal tubular cells to excrete Na^+, thus a contributor of salt-sensitive hypertension.

Since the discovery of the Na/K-ATPase by Skou in 1957 [1], it is well documented that this enzyme is not only the molecular machine for the ATP-dependent and -coupled transport of Na^+ and K^+ across the plasma membrane, but it is also an important signal transducing receptor that capable of bringing both affectors and effectors together to form functional "signalosomes" [2-20].

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1. THE NA/K-ATPASE BIOLOGY

Na/K-ATPase belongs to the family of P-type ATPases and consists of two noncovalently linked α and β subunits [2, 21]. The complete functional Na/K-ATPase contains the "catalytic" α subunit and β subunit which is essential for the assembly of a functional enzyme. Different α and β subunits have been identified and functionally characterized [2, 21]. These subunits are expressed in a tissue-specific manner except that the α1 subunit is expressed in all cells studied. Structurally, both the Na/K-ATPase and the calcium ATPase of skeletal muscle sarcoplasmic reticulum (SERCA) belong to the type-II class of P-type ATPases. Based on the crystal structures of the SERCA1a [22], Sweadner and Donnet [23] first revealed the structural similarities between these two ATPases and concluded that the Na/K-ATPase contains three distinct functional domains, which has been largely confirmed by the crystal structure of the Na/K-ATPase [24]. The actuator (A) domain consists of the N-terminus and the second cytosolic domain (CD2) connected to transmembrane helices M2 and M3. The highly conserved phosphorylation (P) domain includes the parts of the third intracellular loop (CD3) that are close to the transmembrane helices M4 and M5. The highly conserved and relatively isolated nucleotide binding (N) domain contains the parts of the CD3 that are far from the M4 and M5. It appears that the A domain rotates while the N domain closes up during the transport cycle, which opens (E1 state) and closes (E2 state) the A, N and P domains. The A and N domains contain most of the binding motifs in the α1 subunit for its interaction with other partners proteins that have been identified, which are important for the signaling function of Na-K-ATPase. The A domain is able to interact with Src SH2 domain, caveolin-1, PI3K p83α subunit, and ankyrin. The N domain is able to interact with Src kinase domain, PLC-γ, arrestin 2, and spinophilin. These interactions are important for the receptor function and intracellular trafficking of the Na-K-ATPase. A γ subunit (one of the FXYD-containing proteins), which is also expressed in a tissue-specific manner and is not an integral part of the enzyme, may modulate the enzymatic activity [25, 26]. The different functional domains and important binding sites for other proteins are shown in Figure 1 as a cartoon presentation.

Biochemically, the best characterized functional differences between the α subunits are in their relative sensitivities to ouabain [21, 27]. Large differences in ouabain sensitivities are noted across species and different subunits, which are attributed to the dissimilarities in the amino acid sequence [21, 28-34]. The most recognized differences are that rodent α1 is far less sensitive than pig, dog, or human α1; and rodent α2 and α3 are much more sensitive than rodent α1. This notoriously resistant rodent α1 makes it difficult to study ouabain induced α1 inhibition in rodent models. Functionally, as an active ion transporter, the central role of the "ion pumping" Na/K-ATPase is to maintain intracellular Na^+ and K^+ balance and nutrient uptake, and keep other coupled secondary co/transporters alive. In mammalian species, the Na/K-ATPase pumps 2 K^+ ions into the cell whereas it extrudes 3 Na^+ ions from the cells per cycle, against their ion concentration gradient in an ATP-dependent process. The "Post-Albers" model of the Na/K-ATPase "ion pumping" function is shown in Figure 2.

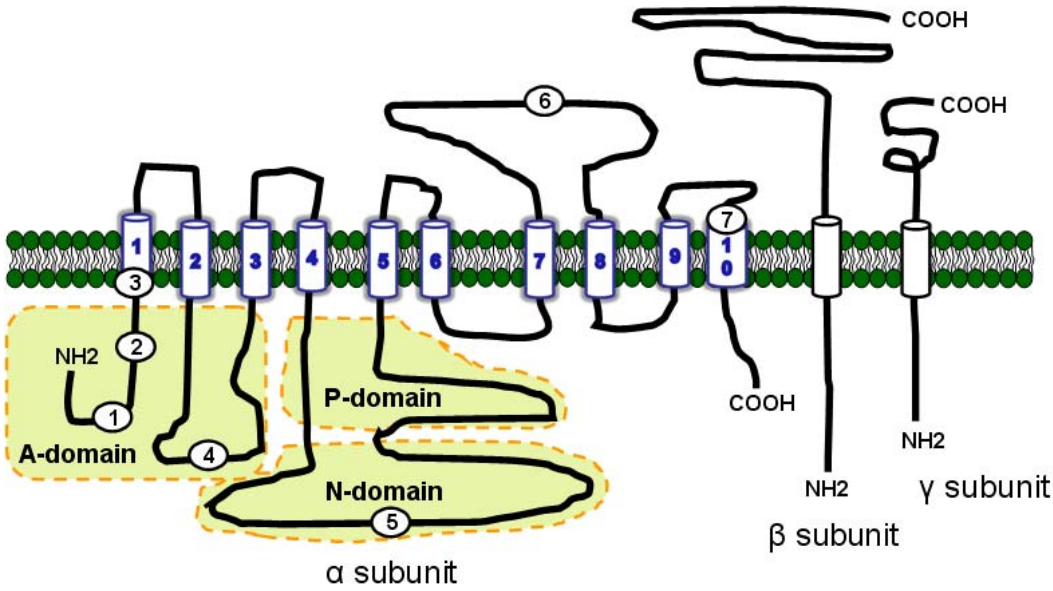

Figure 1. Schematic illustration of Na/K-ATPase and binding sites related to this article. More details please see context. The α1 subunit binding sites for other partner proteins are shown with oval circles with numbers. 1: The LKK sequence is essential for binding of IP3R. The A and L that flanking LKK target the α2/α3 subunits to NCX signaling microdomain in astrocytes; 2: The proline-rich domain (PRD) PxxP binds to the PI3K P85α subunit, which is essential for ouabain and dopamine induced endocytosis of the Na/K-ATPase; 3: The caveolin-binding motif (CBM) FxxF binds to caveolin-1 and keeps the Na/K-ATPase within caveolae microdomain, which is critical for formation of Na/K-ATPase/Src receptor complex; 4: The second cytosolic domain CD2 binds to Src SH2 domain, ankyrin C-terminus; 5: The third cytosolic domain CD3 binds to Src kinase domain, cofilin, arrestins, and spinophilin. 6: The binding of site 6 with extracellular part of the β subunit is essential for full functional Na/K-ATPase; 7: Another CBM that the function is uncertain. Moreover, the 1-85 residues of the α1 N-terminus also bind to 14-3-3ε protein. PKC-dependent phosphorylation of the α1 N-terminal Ser-11/18 by dopamine triggers endocytosis of the Na/K-ATPase.

2. The Signaling Function of the Na/K-ATPase

Other than its "ion pumping" function, recent studies have demonstrated that the Na/K-ATPase also functions as a receptor, signal transducer, and scaffold. Both the A and N domains of the α subunit contain multiple functional motifs that interact with soluble, membrane and structural proteins including Src, caveolin-1, PLC-γ, PI3K, IP3R, adducin, cofilin and ankyrin [15, 35-41]. Binding to these proteins not only regulates the ion pumping function of the enzyme, but it also provides the structure bases for the Na/K-ATPase functioning as a signal transducer. For example, the enhanced interaction between PI3K and the proline-rich domain of the α1 subunit N-terminus in response to different stimuli results

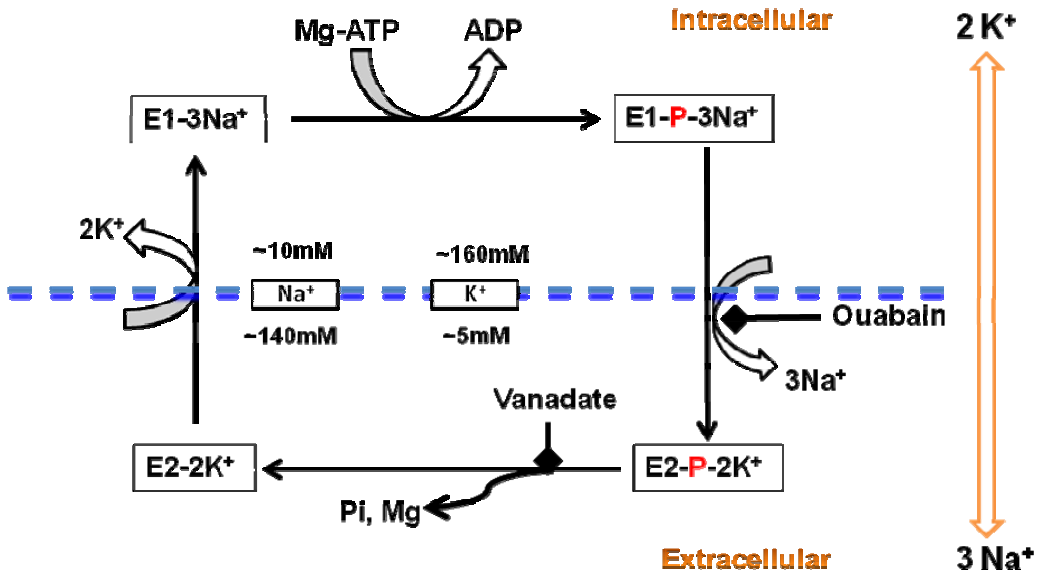

Figure 2. The Post-Albers Model of Na/K-ATPase "ion pumping" cycle. In each pumping circle, two K^+ ions were moved into cells and 3 Na^+ ions were moved out of the cells.

in a cell-specific endo/exocytosis of the Na/K-ATPase and a change in Na/K-ATPase activity [37, 42, 43]. Other ion transporters and channels are also capable of forming signaling complexes and then serve as receptors or as signal integrators that coordinate trans-membrane signal transmission or amplification [44-48].

On the other hand, binding of CTS to the Na/K-ATPase activates Src, and subsequently, activates receptor tyrosine kinases (RTKs) such as EGF receptor (EGFR) and further stimulates serine/threonine kinases, lipid kinases as well as lipases [49]. Specifically, ouabain-induced Na/K-ATPase signaling requires Src in cultured renal proximal tubule cells [10-16, 18, 50-53]. The Na/K-ATPase interacts with Src directly to form a functional receptor complex, and the Na/K-ATPase-associated Src functions as a signal transducer [15, 17]. Multiple domains of both proteins are involved in the direct interaction, with ouabain regulating the interaction between the Src kinase domain and the third intracellular domain of the α1 subunit. A graded α1 knock-down increases the basal Src activity and subsequently tyrosine phosphorylation of FAK (focal adhesion kinase), a Src effector [17]. Concomitantly, it attenuates ouabain-induced activation of Src and ERK. In heterozygous α1 knockout ($\alpha1^{+/-}$) mice, a 30% decrease in α1 expression resulted in more than a 2-fold increase in cellular basal Src activity in the liver [54]. Furthermore, pNaKtide (a 20 amino acid Src inhibitory peptide) is capable of disrupting the formation of the Na/K-ATPase/Src complex and can function as a specific antagonist of ouabain-induced signal transduction in cultured cells and in isolated organs [55].

CTS also stimulate intracellular Ca^{2+} signaling and changes through Src/PLCγ pathway and Na^+/Ca^{2+} exchanger (NCX) coupling [12, 56-59]. Without invoke changes in intracellular Na^+ concentration [5, 60-63], ouabain functions differently depending on the cell type and α isoform. In cells expressing the Na/K-ATPase α2 or α3 (but not α1) isoform, like astrocytes

and smooth muscle cells, Blaustein and colleagues have demonstrated that ouabain induces Ca^{2+} transients through coupling of the store-operated Ca^{2+} channels and/or NCX [62, 63], which is achieved by interaction of the Na-K-ATPase α2/α3 N-terminus with NCX to form a specific calcium-signaling microdomain in many different cell types [38, 64]. In cells expressing only the Na/K-ATPase α1 isoform, like LLC-PK1 and other renal proximal tubule cells, ouabain-induced Ca^{2+} signalings depend on the formation of Ca^{2+} signaling microdomains and direct communication among the Na/K-ATPase, protein kinases and other ion channels/transporters. The binding of ouabain to the Na/K-ATPase not only tethers PLC-γ1 and IP3R together to form a Ca^{2+}-regulatory complex [13] but also stimulates the interaction of the N-terminus of the endoplasmic reticulum-localized IP_3R with the N-terminus of the Na/K-ATPase α1 subunit [5, 13, 39]. Expression of the N-terminus of α1 subunit prevents the interaction between the Na/K-ATPase and IP3R, and consequently abrogates the effects of ouabain on intracellular calcium in LLC-PK1 cells [65]. Interestingly, Ca^{2+} signaling and change have also been shown to regulate NHE3 activity and trafficking [61, 66-68]. A recent report indicates that PLC-γ binds directly and dynamically to the C terminus of NHE3 that is decreased when intracellular Ca^{2+} concentration is increased [69].

Unlike the "ion pumping" function of the Na/K-ATPase, these actions of CTS can occur in the absence of changes in intracellular ion concentrations [11]. Moreover, this Na/K-ATPase signaling function is capable of regulating cell growth in renal epithelial cells, vascular smooth muscle cells and endothelial cells, as well as skeletal muscle cells [3-7, 19, 20, 35, 60, 70-79]. Like other receptors, activation of the Na/K-ATPase receptor complex by CTS induces the endocytosis of this complex [52, 53, 61]. The signaling cascade which depends on caveolar structure and association with Src and the EGFR are contrasted with the "classic" or ionic pathway in Figure 3.

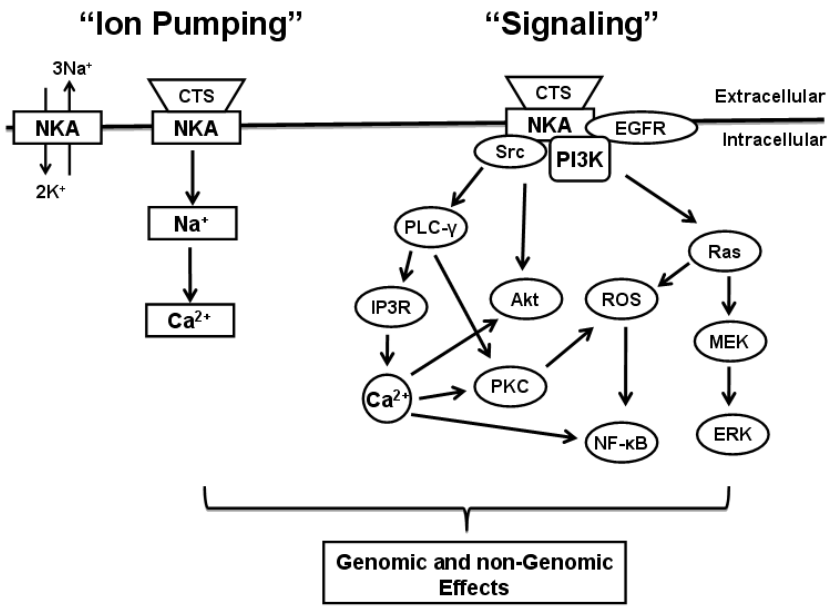

Figure 3. Schematic illustration of the "ion pumping" and "signaling" function of the Na/K-ATPase.

3. NATRIURESIS IN RENAL PROXIMAL TUBULE AND THE ROLE OF REGULATION OF THE NA/K-ATPASE AND NHE3

High salt intake or volume expansion triggers natriuresis by a graded decrease sodium reabsorption in proximal tubule. A defect in such renal adaptation may contribute to salt-sensitive hypertension. In the kidney, the proximal tubule mediates over 60% of the filtered Na^+ reabsorption. It is believed that the apical Na^+ entry through NHE3 is the rate limiting step of Na^+ reabsorption in the proximal tubule because the functional reserve of the Na/K-ATPase in nephron is more than sufficient [80]. In the renal proximal tubule, NHE3 (SLC9A3) resides in the apical membrane, mediating Na^+, HCO_3^-, and fluid reabsorption [81, 82] and plays an important role in the development and control of salt-loading- and volume expansion-mediated hypertension. Up-regulation of the NHE3 activity and expression in the proximal tubule is associated with the development of hypertension [83-87]. Conversely, down-regulation of NHE3 activity and expression occurs during pressure natriuresis in rats [87-92]. NHE3-deficient mice are hypotensive and develop acidosis [93-95]. Via different signaling pathways and partners, the NHE3 activity is regulated via phosphorylation, trafficking and transcriptional regulation [96-100]. The surface expression of NHE3 is mainly regulated by changes in endocytosis/exocytosis, and is considered to be the primary regulatory mechanism of the NHE3 activity [88, 101-113]. Overexpression of the Na/K-ATPase α1 subunit upregulates NHE3 expression and activity [114]. During the development of hypertension in spontaneously hypertensive rats (SHR), the expression and activity of both the Na/K-ATPase and NHE3 are elevated, compared to the normotensive control Wistar-Kyoto rats [83, 115-117]. Under the conditions such as pressure natriuresis, salt loading, and volume expansion, the expression and activity of basolateral Na/K-ATPase and apical NHE3 are coordinately regulated [88, 90, 118-121] to reduce renal Na^+ reabsorption in renal proximal tubule cells. It appears that the pathways regulating the Na/K-ATPase and NHE3 are numerous and redundant. In cases of CTS, PTH and dopamine, they all regulate both the Na/K-ATPase and NHE3 but via different signaling pathways [52, 53, 61, 110, 111, 122-127].

4. ENDOGENOUS CTS AND NATRIURETIC HORMONE

CTS include plant-derived digitalis drugs such as digoxin and ouabain, and vertebrate-derived aglycones such as bufalin and MBG [19, 128]. Recent studies have identified both ouabain and MBG as endogenous steroids whose production and secretion are regulated by multiple stimuli including angiotensin II and adrenocorticotropic hormone (ACTH) [129-131]. The circulating concentrations of CTS were markedly increased under clinical conditions of high salt loading, chronic renal failure, and congestive heart failure [8, 132-139]. Moreover, CTS have many non-cardiac actions [20, 79, 131, 140-143], like the effect on cardiovascular remodeling independent of their effect on blood pressure [9, 14, 76, 77] and on mediation of sympathetic hyperactivity [144, 145].

The essence of the “natriuretic hormone” theory is that, as specific Na/K-ATPase inhibitors, endogenous CTS will rise in response to either a defect in renal Na^+ excretion or high salt intake. The increases in CTS will return Na^+ balance toward normal by increasing natriuresis and cause hypertension through acting on vascular Na/K-ATPase [146]. The

effects of CTS on blood pressure have been demonstrated by their effects on vascular contraction through NCX (reviewed in [56, 147]). However, the pathophysiological significance of endogenous CTS (e.g. as natriuretic hormone) has been a subject of debate since it was first proposed [131, 133, 148] until Lingrel's Laboratory has unequivocally demonstrated that endogenous CTS play an important role in regulation of renal Na^+ excretion and blood pressure through the Na/K-ATPase [149, 150], which confirmed the natriuretic function of endogenous CTS as proposed [146, 151-155]. With a gene targeting approach of knock-out/knock-in transgenic mice ($\alpha1^{R/R}/\alpha2^{S/S}$, $\alpha1^{S/S}/\alpha2^{S/S}$ and $\alpha1^{S/S}/\alpha2^{R/R}$, *S/S* stands for ouabain-sensitive and *R/R* stands for ouabain-resistant), it is clearly demonstrated that CTS-Na/K-ATPase is critical in salt-dependent and ouabain-induced hypertension [147, 156]. In the α1-sensitive ($\alpha1^{S/S}$) mice, there are two very interesting observations: one is that a prolonged inhibition of the α1-, but not the α2-subunit, is compensatory for elevated blood pressure; and the other one is that the α1-sensitive mice show augmented natriuretic response to acute salt-loading and ouabain-infusion compared to α1-resistant ($\alpha1^{R/R}$) mice [150]. These observations suggest that ouabain may regulate renal α1-subunit (causing natriuresis) to counteract its hypertensionogenic effect; and the relative α1 ouabain-sensitivity is a determinant of natriuretic response, which might account for the controversial observations between rodents and other species. Although the ouabain-sensitivity of the α1 and α2 subunits are altered in these transgenic mice, the expression of these two subunits and the total Na/K-ATPase activity in tissues are not affected [149, 157-159]. It is worth to note that CTS are not the sole attributor to the observed diuresis/natriuresis, other regulatory mechanisms (e.g. dopamine and renin-angiotensin-aldosterone system) as well as other nephron segments other than proximal tubules are also involved in Na^+ excretion and reabsorption as well as blood pressure regulation.

Although it remains controversial since the postulation of the existence of a "natriuretic hormone" by inhibiting the Na/K-ATPase about 40 years ago, there is a large body of evidence, including our recent data, supporting the idea that endogenous CTS can physiologically control sodium homeostasis in conditions of chronic renal sodium retention and volume expansion [146, 154, 160-169]. From a classic definition, endogenous MBG might fit the criteria as a putative natriuretic hormone far better than ouabain [167, 170], but endogenous ouabain does have a role in renal adaptation to salt-loading by potentiating natriuresis [130, 167, 171, 172]. Most recently, endogenous ouabain has also been shown as a natriuretic hormone in normal rats [173]. The difference in α1 sensitivity to MBG and ouabain (α1 is more sensitive to MBG than to ouabain) may be attributed to their natriuretic differences in rodents [139]. Unlike MBG and ANP (atrial natriuretic peptide), ouabain-induced natriuretic effect is relatively slow in onset, is sustained, and is not modified by changes in Na^+ status. Additionally, this effect is enhanced dramatically after acute volume expansion, hypervolaemia, small increments in blood pressure, and hypertension [146, 163, 174]. These observations suggest that ouabain-induced natriuretic effect might be secondary to mild hypertension and/or volume expansion, which supports the notion that ouabain, is not a "classically defined" acute putative natriuretic hormone, but acts as a "functional" natriuretic agent.

In retrospect, it may be said that the question was raised far ahead of its time (since the "natriuretic hormone" was proposed to reduce Na^+ reabsorption by inhibiting the Na/K-ATPase only), but now it is appropriate to raise this question again in light of the newly

appreciated ouabain-activated signaling effects (which also regulate apical NHE3). Other than the natriuretic effect, endogenous CTS also function as vasoconstrictors (on vascular α2), which may override their natriuretic effect under some circumstances. This is emphasized by a well known clinical phenomenon: renal vasoconstriction prevents the natriuretic effect of almost any natriuretic agent. This may also explain the paradox seen in severe pre-eclampsia patients, in which treatment with Digibind (an *Fab* antibody fragment that binds to digoxin-like molecules [175] and is commonly used to treat digoxin-toxic patients) appears to cause natriuresis and lower blood pressure [176-178]. Furthermore, diuretic/natriuretic agents are effective antihypertensive agents in a large percentage of patients with essential hypertension [179]. It is conceivable to propose that ouabain may function as both a natriuretic effector and vasoconstrictor, depending on the balance of its overall effects on the vascular α2-subunit (vasoconstriction) and the renal tubular α1-subunit (natriuresis).

Activation of the renin-angiotensin-aldosterone system is known to stimulate sodium reabsorption in the distal tubule and collecting duct [180, 181], a well defined anti-natriuretic pathway. While angiotensin II stimulates release of aldosterone and ouabain in a dose-dependent manner [138], ouabain also stimulates aldosterone secretion [182, 183]. Ouabain has direct natriuretic effect by inhibiting renin secretion without affecting systemic hemodynamics [169, 184, 185], as well as by increasing the pressor effect of norepinephrine, angiotensin, and noradrenaline in normotensives [186, 187]. In low-renin, volume-dependent hypertension, aldosterone antagonists could prevent ouabain-induced vasoconstriction and hypertension [188, 189], and the greatest natriuresis is found in patients with a low plasma renin activity (reviewed in [146]). The cross-talk between CTS and the renin-angiotensin-aldosterone system may have the potential to promote each other's effects.

5. CTS-ACTIVATED NA/K-ATPASE SIGNALING AND ENDOCYTOSIS OF THE NA/K-ATPASE AND NHE3

Recently, the potential physiological significance of the receptor function of the Na/K-ATPase has been demonstrated in regulation of Na^+ handling in renal proximal tubular cells [52, 53, 61, 122, 172]. In LLC-PK1 monolayers grown on Transwell® membrane support, ouabain produced a dose-dependent inhibition of active transepithelial $^{22}Na^+$ flux (from apical to basolateral side). This inhibitory effect occurs only when ouabain is applied at the basolateral aspect of monolayers and the inhibition is fully reversible after cells are extensively washed and fed with ouabain-free medium [52]. This inhibition of $^{22}Na^+$ flux requires ouabain-activated Na/K-ATPase signaling. Specifically, binding of ouabain to the Na/K-ATPase activates Src and PI3K, which subsequently enhances the interaction of the α1 subunit with clathrin and PI3K P85α subunit, and increases the clathrin-dependent endocytosis of the Na/K-ATPase and inhibits its ion pumping activity. The trafficking of the α1 subunit was assessed by both accumulation in early endosome (EE) fraction and decrease in surface expression. Ouabain treatment also compartmentalizes several signaling molecules (Src, EGFR, and ERK1/2) into EE fraction along with the α1 subunit. However, no detectable changes in intracellular Na^+ concentration and total cellular α1 expression were observed. Short term (1h) exposure to ouabain, like dopamine [111, 190, 191], reduces surface expression of NHE3 (assessed by surface biotinylation) and accumulates NHE3 in EE

fraction without affecting total cellular amount of NHE3 protein. Functionally, this results in the inhibition of NHE3 activity measured by either Na^+-dependent pH recovery or $^{22}Na^+$ influx in LLC-PK1 cells [61]. This regulation of NHE3 by ouabain depends on the activation of receptor function but not the inhibition of the Na/K-ATPase because it requires the activation of Src, PI3 kinase and increases in intracellular Ca^{2+}. Moreover, disruption of caveolae structure is also effective in abolishing ouabain-induced NHE3 regulation. Taken together, the above findings indicate that ouabain, like dopamine, can simultaneously down-regulate apical NHE3 and basolateral Na/K-ATPase, resulting in decreases in transepithelial Na^+ transport. Moreover, by following the protocol described by Moe's Laboratory [192], we also found that the recycling of endocytosed surface NHE3 in LLC-PK1 cells was apparently inhibited by ouabain. In addition, "Na^+-clamping" methods indicate that ouabain-induced trafficking of the Na/K-ATPase and NHE3 is independent of intracellular [Na^+] [61]. In agreement with the requirements of NHE3 regulation, ouabain fails to regulate transcellular Na^+ transport when inhibitors of Src kinase and PI3K or intracellular Ca^{2+} chelator BAPTA-AM are used [61]. Taken together, these *in vitro* studies demonstrate that ouabain and other CTS can regulate transepithelial Na^+ transport by controlling the apical NHE3 trafficking and activity via the activation of Na/K-ATPase-mediated signal transduction.

The N-terminus of the Na/K-ATPase α1 subunit contains binding motifs that are critical for ouabain-activated signalings, including the N-terminal CBM that binds caveolin [4], a highly conserved proline-rich domain that binds PI3K [37], and a conserved sequence LKK that binds IP3R [39]. Functionally, we have demonstrated that disruption of caveolae/lipid rafts by Mβ-CD, depletion of caveolin by siRNA, inhibition of Src and PI3K activation, and inhibition of Ca^{2+} signaling all prevent the effects of ouabain on the Na/K-ATPase and NHE3 in LLC-PK1 cells [61, 122]. The relationship amongst the Na/K-ATPase signaling, endocytosis and regulation of the NHE3 are shown schematically in Figure 4.

The α1 Ouabain-Sensitivity

Changing CTS binding affinity of the Na/K-ATPase alters the effects of CTS on renal function *in vivo* [149, 150]. The signaling function of the Na/K-ATPase is largely defined *in vitro* in cultured rat adult cardiac myocytes and LLC-PK1 cells. When over 90% of ouabain-sensitive porcine α1 was depleted and then rescued the Na/K-ATPase-knockdown LLC-PK1 cells with an ouabain-insensitive rat α1 (AAC-19 cells), ouabain-induced activation of Src and ERK cascades occurs at μM concentration in AAC-19 cells instead of nM range observed in LLC-PK1 cells [17]. In three proximal tubule cell lines with different ouabain sensitivities (human HK-2, LLC-PK1, and AAC-19 cells), we found that changes in CTS sensitivity of the Na/K-ATPase α1 also alter the ouabain concentration curve of NHE3 regulation and transcellular $^{22}Na^+$ transport (our unpublished data). Ouabain, at concentrations that did not inhibit the enzymatic activity of the Na/K-ATPase ($^{86}Rb^+$ uptake), inhibits NHE3 activity ($^{22}Na^+$-uptake) and transcellular $^{22}Na^+$ transport, suggesting that the Na/K-ATPase α1 subunit functions as the receptor of ouabain-induced NHE3 regulation, and ouabain-induced redistribution of the α1 subunit and NHE3 is not a species-specific phenomenon.

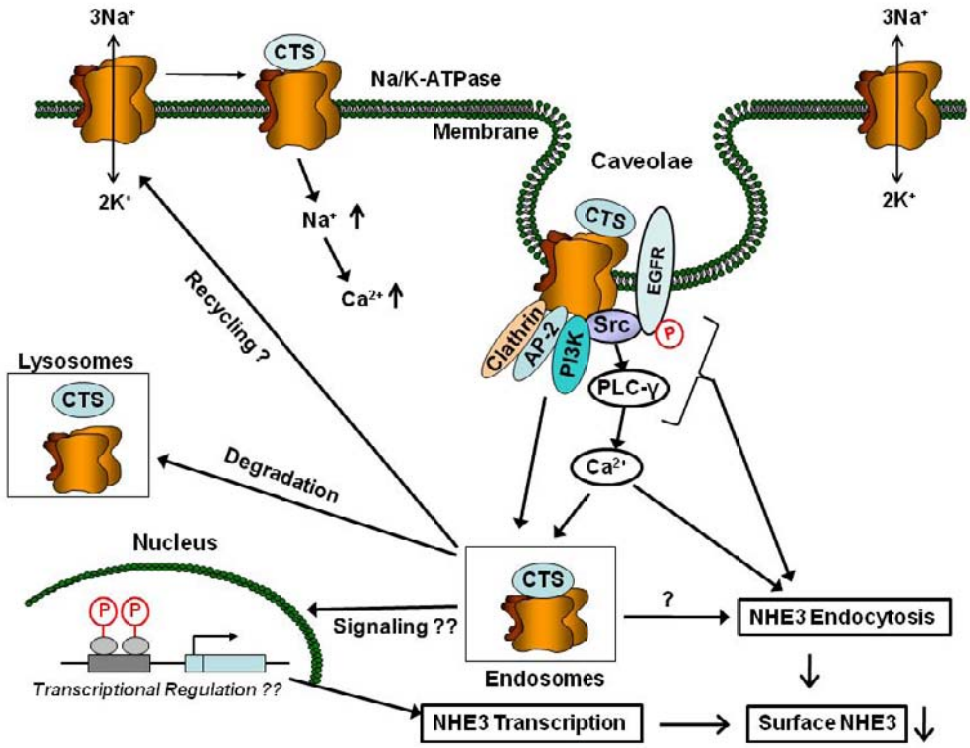

Figure 4. Schematic illustration of CTS-induced regulation of the Na/K-ATPase and NHE3.

Src Kinases

Src family kinases are membrane-associated non-receptor tyrosine kinases. In the resting state, the Na/K-ATPase directly interacts with Src (to keep Src inactive) to form a functional receptor complex. Upon ouabain binding, Src kinase domain is released from the third cytosolic domain (CD3) of α1, resulting in activation of the Na/K-ATPase-associated Src kinase [15-17, 49]. While c-Src-dependent NHE3 exocytosis has been demonstrated by various stimuli [107, 112, 193], c-Src activation is also involved in ouabain-induced NHE3 regulation in LLC-PK1 cells [61, 122]. Inhibition of Src or depletion of c-Src (in SYF mouse fibroblasts [194]) abolishes ouabain-induced endocytosis of the Na/K-ATPase [52]. Despite the possibility of cell-specific response and different stimuli, ouabain and other stimuli may activate different c-Src-dependent signaling cascades. In addition, given that ouabain still binds to the Na/K-ATPase in endosomes and lysosome [52, 195, 196], ouabain-Na/K-ATPase might be able to propagate its original signaling or to initiate distinct endosomal signaling cascades.

PI3K

Ouabain has been shown to activate the p85 subunit of class I_A PI3K and enhance its interaction with the α1 subunit, which is sensitive to Src activation, in both cardiac myocytes and renal epithelial cells [52, 71, 197, 198]. In dopamine-induced internalization of the Na/K-ATPase in renal epithelial cells, dopamine was shown to stimulate the interaction of the PI3K p85 regulatory subunit with the α1 subunit, between the SH3 domain of p85 and a proline-rich domain of the α1 subunit [37]. PI3K is also involved in NHE3 trafficking, including exocytosis, endocytosis and recycling [105, 113, 199, 200]. Inhibition of PI3K activation and its interaction with the α1 subunit prevent ouabain-induced endocytosis of the Na/K-ATPase and NHE3 [52, 61], but the molecular mechanism of PI3K in ouabain-induced NHE3 endocytosis needs further investigation.

PLC/PKC Cascade and Ca^{2+} Signaling

In renal epithelial cells, ouabain-induced Ca^{2+} signaling involves the interaction between the Na/K-ATPase α1 subunit N-terminus with IP3R [5, 13, 39, 65]. Pretreatment with intracellular Ca^{2+} chelator BAPTA-AM abolished ouabain-induced NHE3 endocytosis [61]. While ouabain tethers PLC-γ1 and IP3R together to form a Ca^{2+}-regulatory complex [13], PLC-γ also can bind directly to the C-terminus of NHE3 that is sensitive to intracellular Ca^{2+} concentration [69]. Disruption of PLC-γ-NHE3 interaction prevents carbachol-stimulated, Src- and Ca^{2+}-dependent endocytosis of NHE3 [106]. Activation of PLC and increases in intracellular Ca^{2+} further activate PKC, which can modulate the ouabain-sensitivity and enzymatic activity of the Na/K-ATPase by regulating the phosphorylation status of the α1 subunit [21, 201-203].

Caveolins

Caveolae represent an important signaling microdomain in space for many cellular activities [204, 205]. Caveolins directly interact with many signaling proteins via their scaffolding domains to the CBM of target proteins. In many different cells including cardiac myocytes, smooth muscle cells and renal epithelial cells, the Na/K-ATPase is co-localized with caveolin-1 and concentrated in caveolae [16, 51]. *In vitro* studies indicate that caveolae/caveolins are required for CTS to stimulate the receptor function of the Na/K-ATPase [16, 51]. In LLC-PK1 cells, caveolin-1 interacts with the Na/K-ATPase directly [16, 206] and regulates trafficking of the Na/K-ATPase and NHE3 [53, 61]. Mutation of the N-terminal CBM (F97A and F100A) abolishes the interaction between Na/K-ATPase and caveolin-1 in cultured LLC-PK1 cells [206]. Lipid rafts (and caveolae is a subset of lipid rafts containing caveolins) is not only involved in turnover, trafficking, and membrane delivery of renal epithelial Na/K-ATPase [207], but it also is an important regulatory factor of NHE3 trafficking [208, 209].

We have shown that MBG and ouabain down-regulate the Na/K-ATPase and NHE3 via stimulating endocytosis, and consequently increased urinary Na^+ excretion in rat ([123] and our unpublished data). CTS infusion decreases the proximal tubule surface contents of both

transporters in freshly isolated proximal tubules of Sprague Dawley rats. Concomitantly, significant accumulation of both transporters was detected in the EE fraction. The role of the Na/K-ATPase signaling is intrigued by this observation since rat proximal tubules apparently express caveolin-2, but not caveolin-1 (proximal tubules in other species express both caveolin-1 and 2) [210-212]. Interestingly, caveolin-2 appears to function as caveolin-1 in D_1 receptor signaling in rat tubular cells [213, 214]. Lipid rafts are special membrane microdomains, and caveolae are a subset of lipid rafts with caveolins as structure components and markers. Lipid rafts and caveolae have been implicated in protein trafficking and signal transduction [205, 215]. Caveolae are not absolutely required in signal transduction [215]. For example, G protein-coupled receptor (GPCR) signal transduction could be regulated in both caveolae and non-caveolar lipid rafts [216]. Even though the rat proximal tubule lacks morphologically distinguishable caveolae, the caveolin-2-rich caveolae-like plasma membranes (which are also enriched in the α1 subunit [213]) and/or lipid rafts may function as the regulation sites. While the rat proximal tubular α1 subunit is ouabain-resistant, ouabain-infusion (plasma ouabain in nano molar range) activated the Na/K-ATPase/c-Src complex in isolated renal caveolar microdomains [9] and exogenous ouabain (also in nano molar range) activated ERK1/2 in isolated rat PTs [7].

GPCR (G-Protein–Coupled Receptors) Kinases, Arrestins, and Spinophilin

GPCR kinases (GRKs, like β-adrenergic receptor kinase1 and 2) are capable of phosphorylating the Na/K-ATPase *in vitro* on its CD3 loop between transmembrane domain M4 and M5, which can be stimulated by ouabain. This phosphorylation facilitates binding of the α1 subunit (through the CD3 loop and N-terminus) with arrestins, GRKs, 14-3-3ε proteins, and spinophilin [217]. Overexpression of β-arrestins stimulated the Na/K-ATPase endocytosis while expression of spinophilin appeared to slow the process. Even though ouabain plays a role in this regulation, the effect of CTS-activated Na/K-ATPase signaling is unknown.

Dopamine-Induced Endocytosis of the Na/K-ATPase and NHE3

Dopamine, a well-known natriuretic agent, is the most studied stimulus in endocytosis of the Na/K-ATPase in renal epithelia. The dopamine-mediated Na^+ excretion occurs rapidly and only accounts for about 50% of salt-loading-induced Na^+ excretion [120, 121, 218], suggesting the involvement of other regulatory factor(s). Dopamine-induced natriuresis is mainly due to its capability of inducing coordinated endocytosis of the Na/K-ATPase and NHE3 via D_1-like dopamine receptors and activation of GPCRs [219-225]. Depending on the coupled G proteins, activation of D_1-like receptors may lead to a cAMP/PKA-dependent or cAMP/PKA-independent and PLC/PKC-dependent regulation of the Na/K-ATPase and NHE3. Dopamine-induced endocytosis of the Na/K-ATPase requires the phosphorylation of the α1 subunit Ser-11 or Ser-18 (depending on species) and binding to PI3K p85 subunit, to recruit adaptor protein-2 and clathrin [37, 127, 226-229]. In this process, the 14-3-3ε protein represents a critical linking mechanism for recruiting PI3K to the α1 subunit [230]. The effect

of dopamine on the Na/K-ATPase trafficking seems cell type-specific. In lung epithelia, activation of D_1 receptor by dopamine results in a rapid exocytosis of the Na/K-ATPase from intracellular compartments to the plasma membrane that initiated by phosphorylation of Tyr-5 residue of the α1 subunit and activation of PI3K [43, 231]. Interestingly, it has been shown that dopamine-induced regulation of the Na/K-ATPase in proximal tubules of Dahl salt sensitive rats was defective because of apparent decoupling between the binding of dopamine to its D_1 receptor and activation of GPCRs [232-236]. Dopamine is also able to inhibit apical NHE3 activity by reducing apical membrane NHE3 protein without changing total cellular NHE3 in mouse renal proximal tubule [190]. While dopamine-stimulated NHE3 endocytosis is in a clathrin-dependent manner that requires PKA-mediated phosphorylation of proximal tubule NHE3 and binding of NHE3 with the adaptor protein 2 [111], it has also been shown that dopamine, via D_1-like receptors, inhibits NHE3 activity in rat renal brush border membrane vehicles via both PKA-dependent and PKA-independent pathways [237]. Moreover, activation of PLC by dopamine (increases intracellular calcium and stimulates PKC) is also involved in inhibition of NHE3 activity by promoting its endocytosis [67].

6. PERSPECTIVE

Epidemiological and interventional observations suggest that high dietary salt intake is a key factor in the etiology of human hypertension [238-240]. Arterial hypertension alone, which is intimately linked to excessive salt intake over years, affects about 25% of the adult population in industrialized societies [241]. Kidney is the most important organ in the regulation of Na^+ handling and thus blood pressure, which has now been well documented (for reviews see [242-244]). Recent reports from Lingrel's laboratory clearly demonstrated a specific role of the α isoforms of the Na/K-ATPase and its interaction with endogenous CTS in the regulation of Na^+ excretion and blood pressure in intact animals [149, 150, 157]. The Na/K-ATPase has an ion pumping-independent receptor function that can confer the agonist-like effects of CTS on intracellular signal transduction. Even though it is arguable if such an approach would ever prove more effective than currently available diuretics, the health-related importance of these endogenous factors (as hormones) is self-evident.

It will be of great interest to investigate the role of the Na/K-ATPase in renal sodium handling and blood pressure *in vivo,* such as whether the receptor mechanism of the Na/K-ATPase operates *in vivo,* and whether the stimulation of this receptor mechanism by endogenous CTS induces natriuresis by regulating renal NHE3 activity.

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ABBREVIATIONS

CBM	caveolin-binding motif
CTS	cardiotonic steroids
EE fractions	Rab5- and EEA-1-positive early endosome fractions
EGFR	epidermal growth factor receptor
IP3R	IP3 receptor
MBG	marinobufagenin
NHE3	Na^+/H^+ exchanger isoform 3
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLC	phospholipase C
ROS	reactive oxygen species

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Chapter 3

ENDOCYTOSIS IN UROTHELIAL UMBRELLA CELLS: ENDOCYTIC VESICLES TRAFFIC

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ABSTRACT

Endocytosis is defined as a process where patches of membrane are invaginated and budded off of specialized domains of the plasma membrane. The receptor-mediated endocytosis or clathrin dependant is the most studied mechanism, followed by caveolar and non-clathrin dependant. But in diverse type of cells, such as umbrella cells from the uroepithelium, changes in plasma membrane tension are capable to induce endocytosis. This process occurs by hydrostatic pressure changes during micturion cycle, is clathrin independant and an intact cytoskeleton is necessary. When hydrostatic pressure increases, during urinary bladder filling-phase of micturition cycle, the umbrella cells are stretched and a fusion process of subapical vesicles to apical plasma membrane occurs. After plasma membrane relaxation, during the voiding-phase of the cycle, the additional membrane is endocytosed as discoidal vesicles entrapping the luminal fluid phase. This peculiar endocytosis/exocytosis process, different from the classical endocytosis mechanism, result an appropriate membrane model to study the membrane traffic but also the urinary fluid traffic. The late may have physiophatological implications since the toxins present in the urinary fluid may effectively be delivered to the cell machinery instead to be recycled to the bladder lumen.

1. INTRODUCTION

The epithelial tissues are found in all organs surface. In case of urinary excretion tract, the mucosa surface is covered along the urinary tract from the renal pelvis to the prostatic urethra by a specialized epithelium, denominated urothelium [1]. This stratified epithelium is composed of three cell types: basal cells, intermediate cells and apical or umbrella cells [2]. Figure 1.

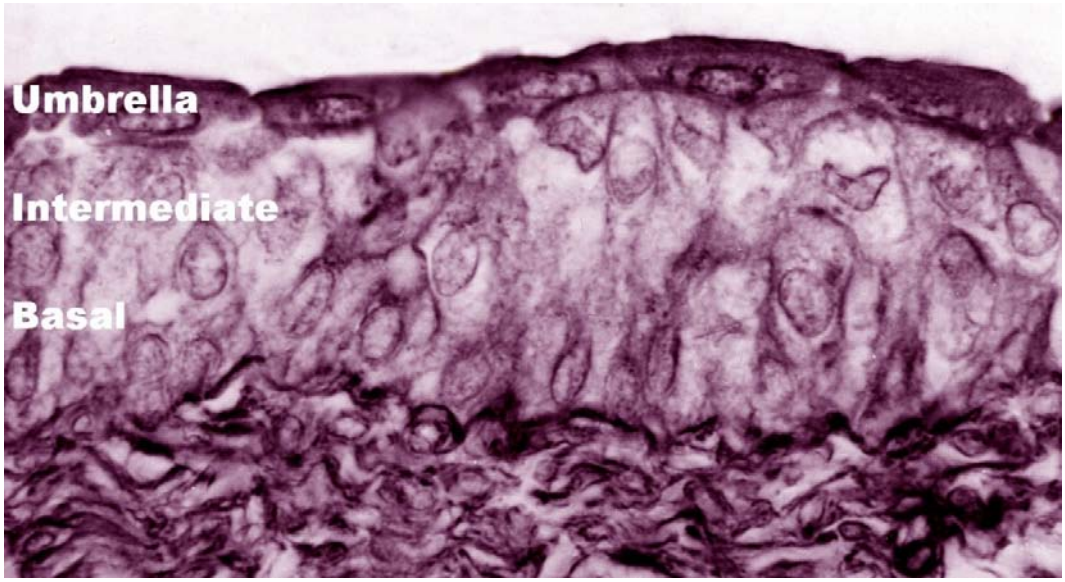


Figure 1. Urinary bladder urothelium. The specific epithelium, urothelium, covers the mucosa surface of the urinary bladder and it is compound of three cell layers; basal cells, which are in contact with the basal membrane, intermediate cells and apical or umbrella cells that are in direct contact with the urine. Image is a courtesy of Dra Mirta Valentich.

Basal cells are small of 10 um diameter and they are in direct contact with the basal membrane, connective tissue and capillary bed. Intermediate cells of 10-25 um in diameter with a pyriform shape and in some species they have a long, thin cytoplasmatic processes that connect them to the basal membrane [3]. The external cell layer which is in direct contact with the urine is composed of large polyhedral cells denominated umbrella cells with diameters of 25-250 um. In some species, such as rats and guinea pigs they present several nuclei. Figure 2. As the intermediate cells, the umbrella cells can extend thin projections connecting the upper cell layer with the basal membrane, [3]. Due to the existence of these projections, some authors have described the urothelium as a pseudostratified epithelium. The umbrella cells have a long live but when damaged, they are rapidly regenerated. The cell replacement is primarily the result of the division and differentiation processes of the basal cells but also, according to Martin, by the fusion of the intermediate cells resulting in a new generation of the umbrella cells [4].

It is widely known that the primary function of epithelial tissues, including the urothelium, is to form a barrier that prevents the entry of pathogens microorganisms (bacterium, fungus and viruses) and selectively controls the passage if water, ions, solutes, and large macromolecules across the mucosal surface of the cell into the underlying tissue. The barrier function of epithelial tissues depends of intercellular union integrity mainly and specialized membrane domains that seal the apical plasma membranes of adjacent umbrella cells [1]. In umbrella cell layer exists high resistance tight junctions that divide the cell surface of these cells into apical and basolateral membrane domains [5]. Another important feature of the apical membrane of umbrella cells is their unique lipid and protein composition which is believed to contribute to the low permeability of this membrane to water and solutes [6, 7, 8].

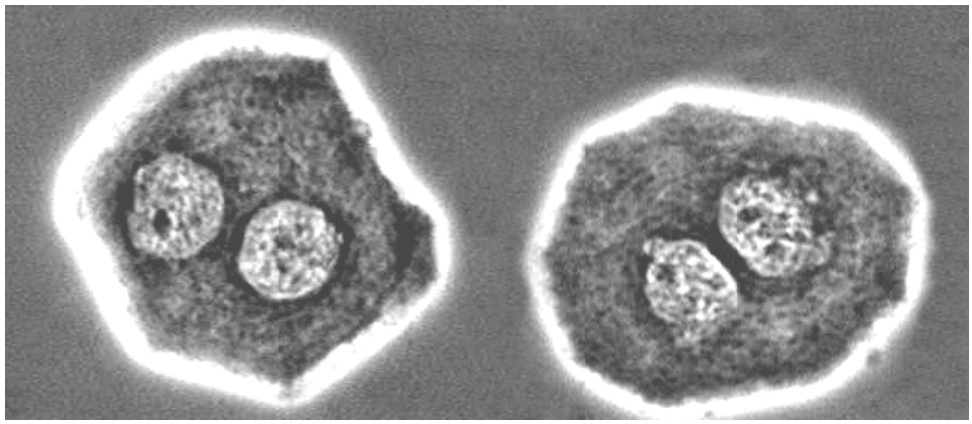


Figure 2. Umbrella Cells. This microphotography was obtained by phase contrast light microscopy. It shows two umbrella cells having their characteristic polyhedral shape. In this case the umbrella cells are multinucleated because they were obtained from rat bladder urothelium.

The epithelial tissues are in many times exposed to mechanical forces. Examples of these are the skin, and the epithelium covering the lumen of the esophagus, rectum and vagina, among others. In case of urothelium, when the urine formed by the kidneys passes to urinary excretion tract, the hydrostatic pressure generated increases, especially in urinary bladder lumen. This pressure increase results in bladder distension and a transition of morphology occurs in all three urothelial layers, especially in umbrella cells layer, where a process of endocytosis-exocytosis of discoidal endocytic vesicles occurs in response to change of mechanical forces [2, 9].

In this chapter we will describe this particular endocytic-exocytic events in umbrella cells layer due to changes in mechanical forces during micturition cycle phases and the pathway followed by the endocytic vesicles. As a new proposal we will discuss how membrane permeability alteration may allow the entry to the cell interior from the endocytosed vesicle lumen.

2. Umbrella Cells Plasma Membrane: Specialized Epithelial Cells

The umbrella cells contain unique structural and biochemical features. The surface of the umbrella cells appears pleated and each cell is surrounded by a tight junctional ring when they are examined by scanning electron microscopy. When high magnification is used, the umbrella cells show raised ridges, also called hinges, which cover the surface surrounding areas called plaques. The association of hinges and plaques results in the characteristic scalloped appearance of the umbrella cell apical plasma membrane, which is well apparent when the apical surface of cross-sectioned umbrella cells is viewed by transmission electron microscopy. Figure 3. The hinge areas actually are not well understood, but contain at least the unique protein called urohingin [10] and presumably all other non-plaque proteins. Plaques occupy approximately 70 – 90 % of the surface of the umbrella cell.

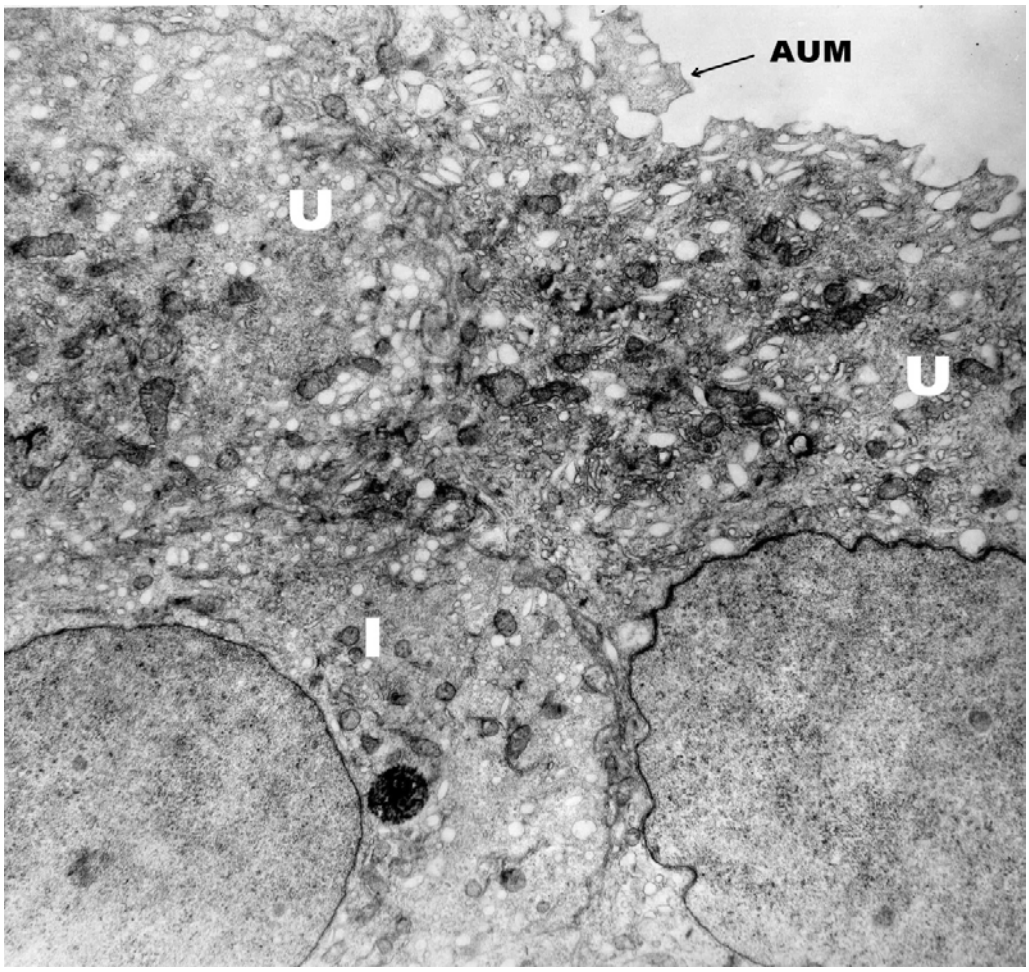


Figure 3. Ultrastructure of the umbrella cell. At higher magnification of umbrella cells we can see their characteristic Asymmetric Unit Membrane (AUM) that is twice thicker in its luminal side (arrow). Beneath the AUM there is a pool of endocytic vesicles having a membrane reservoir role to the apical plasma membrane. The image of rat ureter is a courtesy of Dra Mirta Valentich. U: umbrella cell, I: intermediate cell.

Liang *et al*. by using sarkosyl, a harsh detergent, obtained a pure plaques fraction containing also the hinge areas. The highly detergent insolubility [11] of this luminal membrane may reflect the unusual lipid composition of this membrane, which is rich in cholesterol, phosphatitylcholine, phosphatitylinositol, phosphatityl ethanolamine and cerebroside, a lipid profile similar to myelin [6, 7, 8]. Keterer *et al* reported the presence of saturated and polyunsaturated fatty acid n-6 derivatives mainly and high levels of one eicosatrienoic, acid marker of essential fatty acid deficiency, unusual lipid in normal tissues [12].

Another important characteristic of the apical plasma membrane of umbrella cells is that the outer leaflet appears to be twice as thick as the inner leaflet, thus forming an asymmetric unit membrane (AUM) [13]. When negative staining solubilized membranes, of the AUM were observed by high resolution microscopy and by quick freeze, deep-etch techniques a well-developed paracrystalline array of 16 nm diameter AUM particles was apparent. Figure

4. This paracrystalline particle array presents six-fold symmetry, forming a twisted ribbon structure composed of an inner ring containing six large particles and an outer ring containing six small particles [13]. AUM particles protein constituents are the uroplakins, a group of at least five proteins including the tetraspan family members UPIa (27 kDa) and UPIb (28kDa), and the type I single-span proteins UPII (15 kDa), UPIIIa (47 kDa) and UPIIIb (35 kDa) [14]. Uroplakins Ia and Ib play multiple roles in cell migration, cell signaling, viral infection and membrane architecture [15]. The UPIa also serves as a receptor for the uropathogenic Escherichia coli [16] which represents more than 90 % of the urinary tract infections. An exceptional feature of Uroplakin IIIa is it has a 50-amino acid-long cytoplasmic domain, which is believed that mediates the interaction between the urothelial plaques and underlying cytoskeleton [17]. Uroplakin IIIb is a minor protein found in urothelial plaques, of which the specific role is not known [18]. We described for the first time the dependence of AUM particles symmetry with the membrane lipid composition, which by modifying the membrane fatty acid composition resulted in changes of the relative amount of uroplakin dimmers [19]. In effect we have shown by chemical "cross-linking" an increase of the heterodimer UPIb/UPIII and a decrease of the homodimer UPIII/UPIII in the olein diet derived urothelium. From those observations we inferred that the possible lipid-protein alteration may be the cause of the altered uroplakin dimmers reported by us [19]. In accordance with those data we observed a statistically significant increase of the particle size from 15 nm (control AUM particles) to 17 nm center-to-center particle in an olein diet derived urothelium [20].

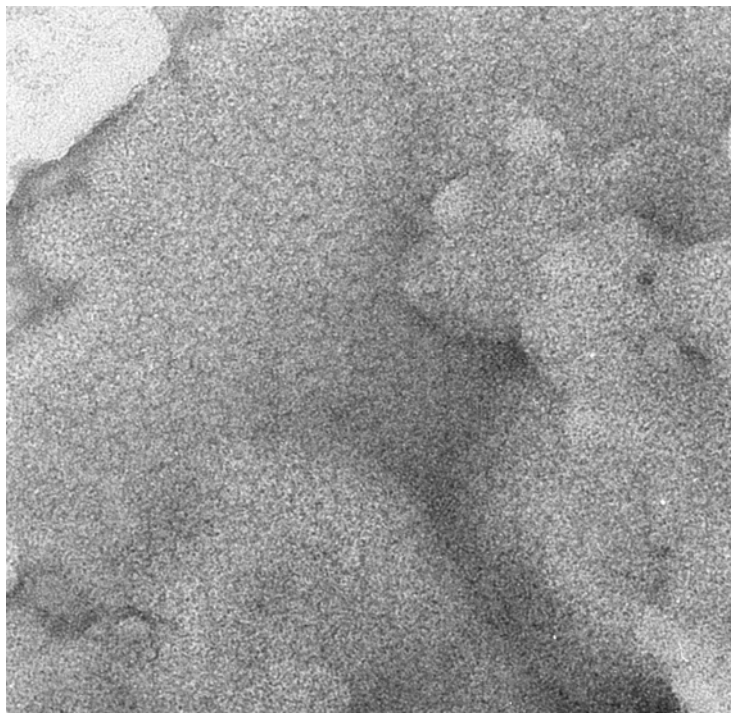


Figure 4. Urothelial Particles: this is a microphotography of a negative stained urothelial plaque. Th is is covered by the 17 nm diameter AUM particles. These particles are composed by the uroplakins imbibed in a lipid matrix. The particles present six-fold symmetry forming a twisted ribbon structure.

UPIa, UPII, UPIIIa and UPIIIb are only expressed in the urothelium and are concentrated in the umbrella cell layer [18]. Uroplakin Ib besides to be an AUM constituent is present too in cornea, conjunctiva, placenta, and kidney and it may have a role as a protection barrier of the ocular surface from bacterial infection [21] like in urinary bladder,

In addition to the specialized AUM in apical membrane, the umbrella cells are connected each others with well-developed claudin-8-positive tight junctions, which polarizes the umbrella cells in two different domains: apical and basolateral [22]. Beside the mentioned apical membrane characteristic and its characteristic subplasmalemal vesicles, the basolateral membrane is highly folded as a membrane reservoir.

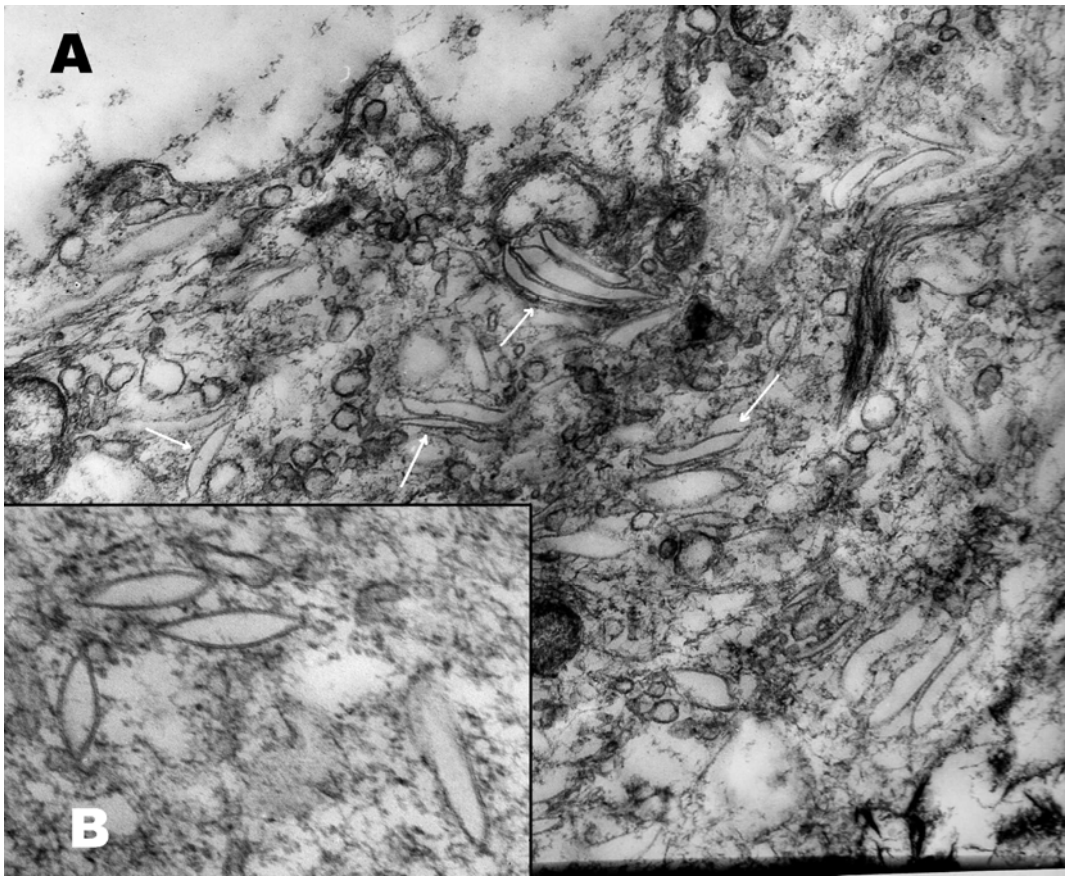
3. CYTOSKELETON AND PLASMA MEMBRANE DYNAMICS RELATED TO THE VESICLE RECYCLING PATHWAY

The umbrella cells contain a high density of cytoplasmic vesicles. Figure 5. These vesicles are composed of two opposing plaques joined together by hinge membrane [23]. Hicks [24] reported that the cytoplasmic vesicles are associated with intracellular filaments. Staehelin *et al.* [25] observed that the cell cytoplasm of umbrella cells contained a dense network of filaments and proposed that the plaques are connected to these filaments by short, cross-linking filaments. Coincidently, Minsky and Chlapowski [26] proposed that the surface plaques and cytoplasmic vesicles are interconnected via the filaments. Moreover, they proposed that these filaments attach to the tight junctions between the lateral and apical membrane and to the desmosomes in the basolateral membrane of the umbrella cells.

There is several data that demonstrates a highly developed cytoskeleton structure in the urothelium [24, 25, 27] and Kamada *et al.* have classified the cytoskeletal structure into micro (4-5 nm in diameter) and intermediate (6-10 nm in diameter) filaments [28]. Moreover they described the protein composition of the cytoskeleton filaments [29]. Actin was found to be the predominant cytoskeleton component beneath the apical plasma membrane. The hexagonally arranged intramembranous particles in the plaque regions are thought to function as anchoring sites for the actin filaments and that may serve to prevent the rupture of the plasma membrane when the bladder is distended by the increase in hydrostatic pressure during filling phase of micturition cycle. In addition, the formation and movement of endocytic vesicles in the contracted bladder has also been attributed to the dense network of cytoskeleton elements [29, 30].

The microfilaments were observed as active components involved in motile functions such as cytoplasmic streaming, cell division, secretory processes, among others in nonmuscle cells [31, 32]. In case of umbrella cells the contractile activity has been suggested due to the existence of actin filaments and Ca-ATPase activity under the luminal plasma membrane, which could be a possible contractile system when they interact with myosin molecules [28, 33].

The distribution of intermediate filaments in the cytoplasm of the umbrella cells appears to correspond to the tonofilaments which consist predominantly of the cytokeratin variety measuring approximately 6-10 nm in diameter [29, 34]. Normally the bovine urothelium synthesizes keratins K8, K18 and K19, with traces of K5 and K6 [35, 36, 37].



The image was courtesy of Dra Mirta Valentich.

Figure 5. Urothelial Endocytic Vesicles. A) The umbrella cells have a cytosol pool of endocytic vesicles. These vesicles have a role of membrane reservoir for the apical plasma membrane that is subjected to a tension increase during the filling phase of the micturition cycle. During the voiding phase of the micturition cycle, the added membrane is endocytosed, forming the vesicles, entrapping the luminal fluid phase. A synthesis de novo is recently suggested that also restores the vesicles population. Arrow: endocytic vesicles. B) Four endocytic vesicles where is well apparent the Asymmetric Unit Membrane (AUM).

Kamada et al observed the intermediate filaments in the form of bundles or dense networks throughout the cytoplasm forming a dense meshwork oriented in parallel and at right angles to the undersurface of the plasma membrane [29].

Sarikas and Chlapowski [38] have shown that disruption of the intermediate filament network with 5 mM thioglycolic acid results in the blocking of endocytic vesicles traffic toward and from the apical plasma membrane. During contraction, they found that the superficial, umbrella cell, and the intermediate cell interface unfolded while the apical plasma membrane surface ballooned out into the bladder lumen. Large intercellular spaces were formed in expanded bladder between superficial cells along their lateral and basal margins. They believed that the loss of intermediate filaments precludes events which usually accommodate the transition in cell shape, such as normal surface area changes due to endocytosis and exocytosis in the apical membrane as well as cyclic folding and unfolding of lateral and basal membranes. From this evidence it has been suggested that cytokeratin

filaments support the plasma membrane to external pressures during the expansion-contraction cycle by forming important linkages to the cell-cell adhesion systems [39].

Can the Endocytic Vesicles Traffic Relay on Cytoskeleton as the Main Regulator Factor?

Indeed the effectiveness of endocytic vesicles traffic do not relay by complete on cytoskeleton. We cannot rule out the role of the membrane lipid dynamic properties which may induce the cytoskeleton reorganization and/or allow the necessary physic phenomenon of lipid membrane redistribution implicated in any process of membrane fusion or membrane translocation. In the fusion process membrane lipids have a critical regulatory role; for instance, ordered lipids domains have the ability of exclude some proteins related in fusion phenomenon [40]. Several reports exist indicating the protein function dependency on their "surrounding lipidic environment". In order to do not exceed the limits of this chapter we will not extend on this matter.

It is remarkable that membranes do not fuse each other unless a highly curved membrane region is allowed to form [41]. It is well know that negatively curved phospholipids enriched liposomes undergo spontaneous fusion, whereas incorporation of positively curved lipids hinders fusion [42]. Lipids that provide negative curvature are phosphatidylethanolamine, cholesterol, diacylglycerol and phosphoinositides, all presents in AUM and endocytic vesicles plasma membrane [43, 7].

Generally the membrane fusion involves tethering and docking, priming and fusion of the membranes finally [44, 45]. Tethering and docking occurs in a specific region of various membrane compartments and in this docked state the membranes can receive the signals that undergo fusion [44]. In order to the fusion phenomenon occurs, it is necessary precise membrane proximity and high differences in density of the adjacent membranes. The membrane fusion is promoted by differences of lipid phase transitions, lateral phase separation or domain generation of the membranes involved in fusion phenomenon [46]. *Moreover, osmotic, electrical and mechanical stress besides high local membrane curvature can induce membrane fusion phenomenon* [41, 46]. Knowing the differential lipid molecular shape and their critical packing parameters it becomes evident that the lipid membrane composition may affect the mechanical properties of the biological membranes. Thus phosphatidic acid, phosphatidylethanolamine and phosphatidylserine can adopt the cone shape favoring the negative curvatures, while lisophospholipids adopt the inverted cone shape leading to the positive curvature. Thus, liposomes enriched in negatively curved phospholipids undergo spontaneous fusion, whereas incorporation of positively curved lipids hinders fusion [42]. Membrane lipid composition can change by endogenous lipid metabolism alteration as well as by signal transduction response triggered by external signals. Such as the case of the activation of phosphatidylcholine-specific phospholipase, PLD2. This plasma membrane enzyme through the generation of lipid signaling molecules as phosphatidic acid and diacylglicerol may induce cytoskeletal reorganization and subsequently, changes in cell morphology and intracellular vesicle movement [47].

In case of chromaffin cells plasma membrane, the phospholipase D1 generates phosphatidic acid that regulates exocytosis. This phosphatidic acid generates a negative

curvature and thus promotes fusion of secretory vesicles with the plasma membrane [48]. Furthermore, the neurons have a presynaptic protein - endophilin - that binds to the GTPase dynamin implicated in endocytosis and recycling of synaptic vesicles. This endophilin has lysophosphatidic acid acyl transferase activity which will transfer the unsaturated fatty acid arachidonate to lysophosphatidic acid and thus converting it to phosphatidic acid. This lipid composition alteration led to a lipid shape change acquired by converting an inverted-cone shape lipid molecule to a cone-shape lipid in the cytoplasmic leaflet resulting in a negative membrane curvature. This is the requirement for the synaptic vesicles formation and the ulterior fusion of the synaptic vesicle to the plasma membrane [49].

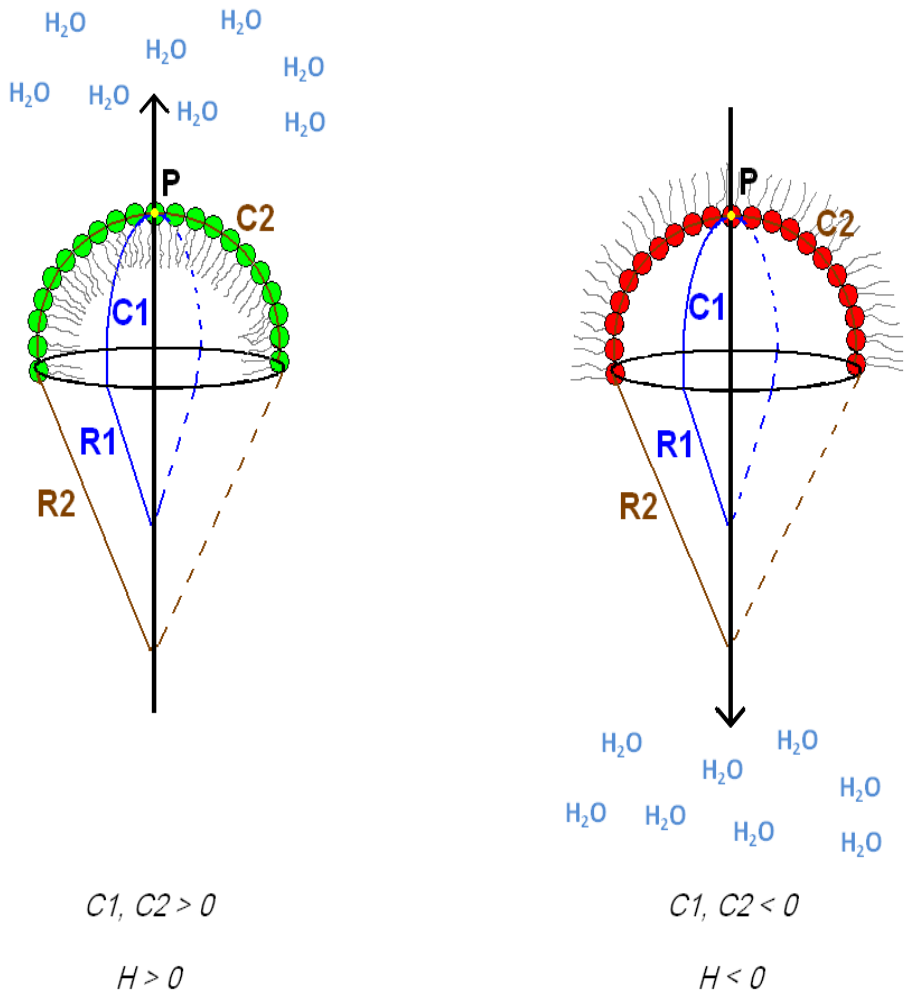
Cholesterol also has effects on fusion processes such as SNARE complex formation, hemifusion, pore formation and pore dilation [50]. This lipid is required, after Ca^{2+} triggered, to enable fusion of secretory vesicles to the plasma membrane. These fusions processes seem to be mediated by the ability of the cholesterol to produce spontaneous negative curvature where cholesterol-sphingomyelin enriched microdomains organize and regulate the fusion process [51].

To undergo fusion between the neurons plasma membrane and synaptic vesicles, it is necessary the interaction of t-SNAREs and v-SNARE present in opposing membranes. This forms ring-complexes, in presence of Ca^{2+}, where exist continuity between the opposite membranes. The critical size of the t-/v-SNARE ring complex is determined by the curvature of the membranes and again the critical presence of lipids allowing the shape change will be required [52].

Also the amphipathic alpha-helical peptides have the ability to induce curvature stress in lipid membranes. They can directly deform the membrane or promote the formation of defects that involve highly curved lipid layers present in membrane pores, fusion intermediates, and solubilized peptide-micelle complexes [53].

Briefly we should consider *what the plasma membrane curvature is.* We usually describe the plasma membrane as a flat structure in order to have a better and simple model to study it. But, the cell plasma membrane is not a flat structure. In fact it has a curvature determined by the packing and interactions of its molecular constituents, especially the lipids, which compose the plasma membrane [54]. This curvature can be positive when there is a convexity of the bilayer to the aqueous phase. Instead, the plasma membrane curvature is negative when the bilayer is concave to the aqueous phase [55]. Figure 6.

The AUM and plasma membrane of urothelial endocytic vesicles are composed, among other lipids, by phosphatidylethanolamine, cholesterol, diacylglycerol and phosphoinositides, all lipids that induce negative membrane curvature and thus promoting the fusion between the AUM and the endocytic vesicles [7, 43]. Figure 7. Moreover we found a high content of phosphatidylserine and phosphatidylethanolamine in the urothelial endocytic vesicles [8]. It is known that phosphatidilserine is involved in fusion phenomenon [56]. Furthermore, when associated to the inverted hexagonal phase (H_{II}) promoter phosphatidylethanolamine, which induce packing stress affecting, for instance, protein membrane conformation and topological changes required in fusion phenomenon [57].



This figure was adapted from [55].

Figure 6. Plasma Membrane Curvature: the plasma membrane curvature can be defined by drawing two perpendicular lines at a given point (P). The curvatures of the two lines are the principal curvatures (c_1 and c_2). Because the membrane surface is almost circular proximal to the interceptions lines, the radii of these two circular fragments, R_1 and R_2, are defined as the principal radii of curvature, and their inverse values are referred as the two principal curvatures c_1 and c_2. Plasma membrane curvature is positive when there is a convexity of the bilayer to the aqueous phase with positive values of principal curvatures c_1 and c_2 and the Gaussian curvature H. On the contrary, the plasma membrane curvature is negative when the bilayer is concave to the aqueous phase and the values of the curvatures c_1, c_2 and H are all below 0.

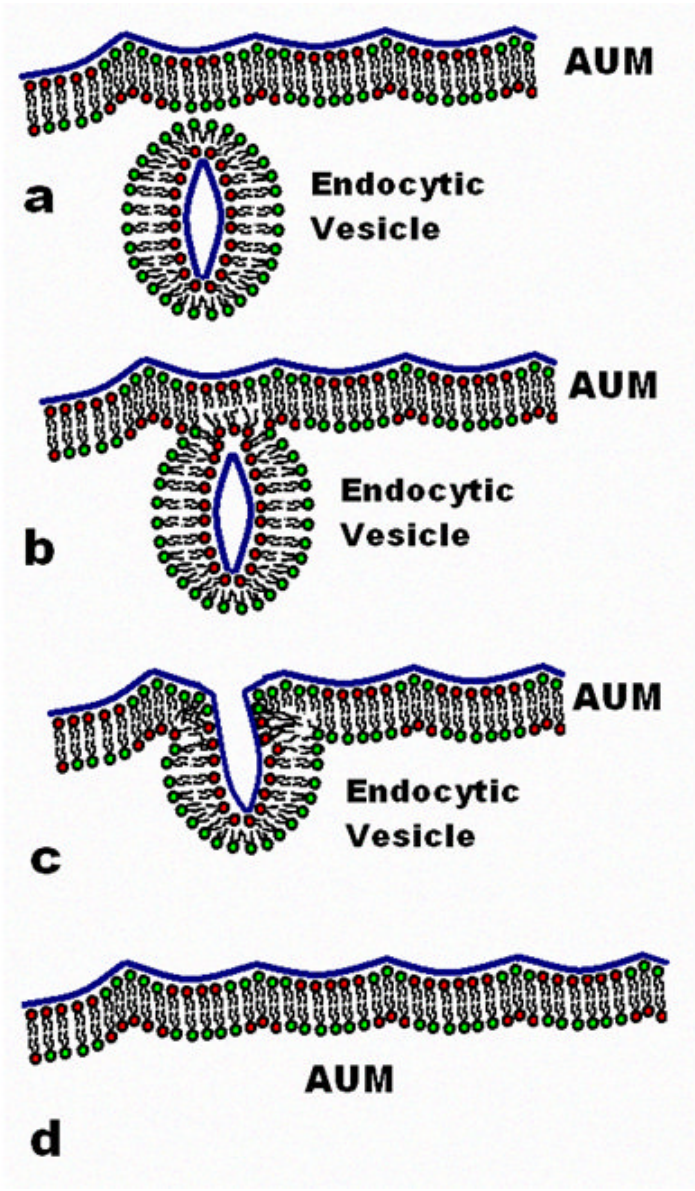


Figure 7. Plasma Membrane Fusion. Negative curvature of the plasma membrane undergo fusion more easily that positive curvature. The umbrella cell plasma membrane and endocytic vesicles are composed by cholesterol, phosphatitylcholine, phosphatitylinositol, phosphatitylserine, all lipids that induce negative curvature. In this model we describe a simple fusion phenomenon between an endocytic vesicle and the apical plasma membrane. The lipids with red polar head have negative curvature and the group with green polar head has positive curvature. a. the endocytic vesicle is proximal to the apical plasma membrane, b. the lipids are highly dynamic and fusion phenomenon begins. c. the vesicle is partially fused to the apical plasma membrane, and the urothelial particles (blue thick continuous line) composed of uroplakins mainly are transferred to the asymmetric unit membrane (AUM). d. the vesicle has fused increasing the apical membrane surface.

4. PLASMA MEMBRANE TENSION

When a cell modifies its surface and area, it is not a passive mechanism. Indeed most cells use endomembrane reserves to continually reordering their plasma membrane to maintain a given volume or shape [58]. Membrane homeostasis, tension regulation, area regulation and mechanosensitive membrane traffic are all used to describe membrane-reservoir exchange [59]. The umbrella cells, when exposed to increased tension, by the increase of hydrostatic pressure of filling phase, increase their area by incorporating new membrane material from the endocytic vesicles. Conversely, when the tension is sufficiently low, they may delete material from the membrane, by endocytosis of the added membrane, and thus regain their original shape [2, 5, 9].

But the modifications in surface and area results in changes of Tension that is one intrinsic feature of the plasma membrane [60] and has a significantly impact on endocytosis and exocytosis. Almost all cells constantly adjust the amount of lipids in their plasma membrane in order to maintain its lateral tension at some specific set point [61, 62]. Morris and Homann developed the Tension Hypothesis for Surface Area Regulation: *When membrane tension goes high locally, Surface Area is added locally for widespread, mechanically accessible endomembrane reserves. When tension goes low locally, excess Surface Area is retrieved locally* [58].

Actually we can quantify membrane tension using laser tweezer tether force. The laser beam of the tweezers propagates through an optical medium with Kerr (focusing) and higher order (defocusing) nonlinearities and displays pressure and surface-tension properties yielding capillarity and dripping effects totally analogous to usual liquid droplets [63]. A bead is bounded to the plasma membrane, and then it is trapped by a laser tweezer and pulled away from the cell at a constant velocity. Figure 8. The tether force can be calculated by measuring the bead displacement from the center of the laser (Δd) and calibration to the trap. Then membrane tension can be calculated from the tether force [64, 65, 66]

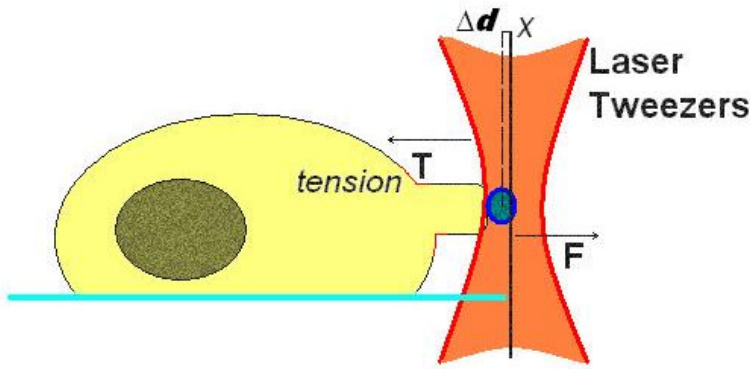


Figure 8. Measure of plasma membrane tension using a bead attached to laser tweezers. A bead, i.e. an antibody, is docked to a plasma membrane tether and then pulled away (F) by the tweezers a constant velocity. Once reached the maximum stretching, the tethering force (T) is measured by the displacement of the bead from the center of the laser (Δd). Using the tether force is possible to calculate the membrane tension [60, 65, 66].

The in-plane tension of a cell plasma membrane is more complicated to discern when the plasma membrane is attached to the underlying cytoskeleton, and this adhesion contributes significantly to the apparent membrane tension [66, 67]. Other factors affecting plasma membrane tension include hydrostatic pressure across the membrane and effects due to local membrane curvature such as regions of membrane associated with microvilli where the membrane area is increased in a small surface [66].

In case of bladder urothelium, the increase of hydrostatic pressure arises plasma membrane tension of umbrella cells mainly, and undergoes an exocytosis of endocytic vesicles as a response to the increased tension [2, 5, 9].

5. MICTURITION CYCLE: UROTHELIUM RESPONSE TO BLADDER FILLING AND VOIDING

When the urinary bladder fills with urine, the hydrostatic pressure rises in a tri-phasic manner. In the first phase, the storage phase, the pressure rise occurs rapidly and then remains relatively constant for an extended period of time. The storage phase is followed by the micturition or urine elimination phase, characterized by a rapid rise in bladder pressure due to smooth muscle contraction. Upon voiding, the pressure returns to baseline and the process begins again [68].

The increased urine volume is accommodated in two ways by the urothelium. The principal mechanism is the unfolding of the mucosal surface, which is highly wrinkled in the empty bladder. The other mechanism occurs at the cellular level and involves a transition of the morphology and function of the urothelium. As the urinary bladder fills, the urothelium becomes thinner, as result of intermediate and basal cells being pushed laterally. Umbrella cells undergo a large progressive shape change from a roughly cuboidal morphology, in the empty bladder, to a flat and squamous shape in the filled urinary bladder [23].

Are There Changes in the Membrane Reservorious during the Micturition Cycle?

It is known that fluids do not have elasticity neither rigidity, and because a lipid bilayer has a degree of both, we can consider it in some aspects as a solid [64, 69, 70, 71]. Artificial bilayers and biological membranes stretch elastically until their area increases by 2-3 % maximum at 1-12 mN/m tension. If tension exceeds this limit the membrane will colapse [58].

During the filling phase the increasing urine volume raises the hydrostatic pressure, and because the plasma membrane is a poor elastic structure (at this point the membrane tension increase is critical), it results in exocytosis of the endocytic vesicles located beneath the apical plasma membrane of umbrella cells. As consequence of this, the apical surface of umbrella cells is increased. Truschel et al. demonstrated by stereology and estimates of apical membrane capacitance (where 1μF ≈ 1cm2 of surface area) in isolated tissue that increased hydrostatic pressure stimulates a 50 % increase in apical surface area that is coupled with a significant decrease in vesicle surface area [72].

Normally in the storage phase when the hydrostatic pressure reaches a maximum, the micturition act occurs. Upon bladder voiding the plasma membrane tension decreases and the apical membrane added during filling is rapidly endocytosed entrapping the luminal fluid phase. This is supported by microscopical analysis where much less endocytic vesicles were visualized in filled bladders [2]. As mentioned above, all this endocytosis – exocytosis traffic of vesicles need an intact cytoskeleton.

However, recent data suggest that endocytosis can occur also during the filling phase of micturition cycle. Thus, Truschel et al. [72] reported two inconsistencies of the data referred to the classical model of vesicles endocytosis – exocytosis described so far. First, the amount of membrane added to the apical membrane of umbrella cells when the epithelium is exposed to pressure (~ 1500 μm^2) is significantly less than the amount of membrane present in the steady – state pool of discoidal vesicles (~ 7000 cm^2). Second, the total amount of cell associated uroplakin UPIII decreases significantly after exposing the uroepithelium to pressure for 5 hours. This puzzling question was solved when biotin-labeled surface protein assays were used to demonstrate that increased hydrostatic pressure not only stimulates exocytosis, but also stimulates rapid endocytosis [72].

6. FATE OF ENDOCYTIC VESICLES: NEW ALTERNATIVE PATHWAYS

Hicks proposed a model of endocytic vesicles traffic that for many years satisfied all research on urothelium [2]. In fact the vesicle traffic was conceived as a mere membrane reservoir. Thus, once endocytosed, the vesicles were located beneath the apical plasma membrane of umbrella cells "waiting for" the increase of membrane tension of the filling phase of the successive micturition cycle and consequent exocytosis of them. But recent data propose new alternative pathways of endocytosed material, plasma membrane and luminal fluid phase. Figure 9. Zhang *et al.* [73] founded AUM in multivesicular bodies and also in autophagosomes. They injected Horseradish Peroxidase (HRP) in resected bladders and the tracer was distributed on the free surface of the superficial, umbrella, cells. No positive structures were found in the cytoplasm at this time. At 30 minutes after the HRP injection, endocytic vesicles containing HRP were observed in the cytoplasm beneath the umbrella cells apical plasma membrane. After 1 and 2 hours of HRP administration the endocytic vesicles had moved to deeper portions of the cytoplasm [73]. Some of them were enlarged and rounded and inclusion vesicles appeared in their lumen, as if they were beginning to transform in Multivesicular Bodies. Some others got access to lysosomes.

Truschel *et al.* [72] also found new evidence that once endocytosed, the vesicles could be degraded via lysosome. After the apical surface of umbrella cells was biotin-labeled, they induced endocytosis of the labeled plasma membrane in a time range of 5 – 300 minutes. After each time point the proteins were analyzed by inmunoblott. The total amount of biotinylated proteins remained constant for up to 120 minutes. After 210 minutes they found a significant decrease in protein to ~ 20 % of the starting value and by 300 minutes the protein levels were reduced to ~ 9 %. Two plausible mechanism of the protein decrease were examined: the lysosomal and proteasomal degradation. To discriminate between the two mechanisms, Truschel *et al.* block first the lysosomal degradation and after that, the

proteasomal. Only when blocked the lysosomal degradation the biotinylated proteins remains constant, indicating the followed pathway of endocytic vesicles.

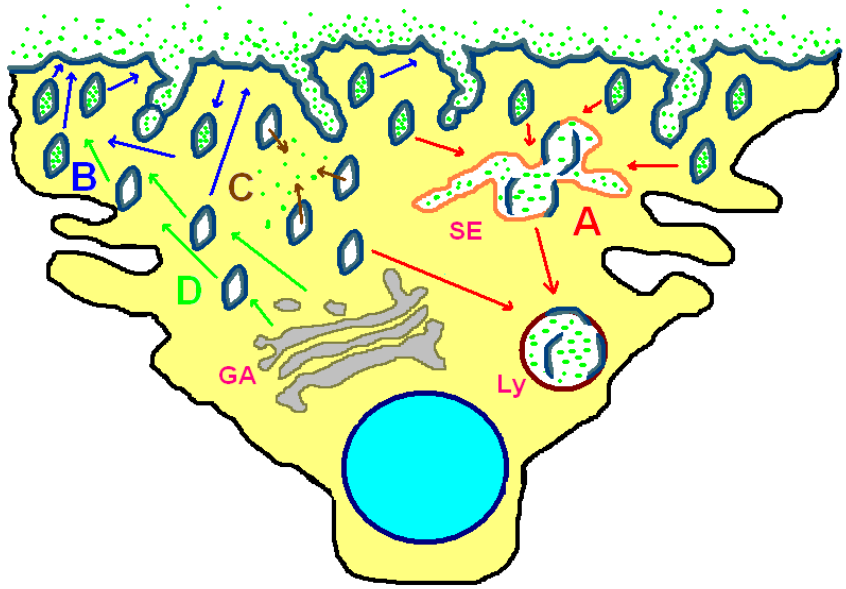


Figure 9. Endocytic vesicles pathways. Four routes are possible: A: once endocytosed the vesicles could be directed to the sorting endosome (SE) and after that delivered to the lysosome for degradation. B: the clasical recycling pathway where the endocytic vesicles are exocytosed to the luminal surface. The third route (C) is the release of the content to the cytoplasm from the vesicular lumen. A novo sinthesis of vesicles probably exists restoring the vesicles pool beneath the apical plasma membrane (D).

Indeed endocytic vesicles have an acidified lumen as well as a GTPase Rab27b [74, 75] which participates in targeting the endocytic vesicles to lysosomes. They also present Vps33a, a Sec-1 related protein implicated in vesicular transport to the lysosomal compartment [76]. This result is reinforced for studies with a mutation of Vps33a in mouse which causes the Hermansky-Pudlak syndrome, characterized by oculocutaneous albinism and bleeding, due to traffic alterations of melanosomes and platelets respectively of lysosome-related organelles [75, 77]. However the endocytic vesicles are CD63 negative, a tretraspanin protein known as a lysosome-related organelle marker [78], and therefore they are not typical lysosome-related organelles.

We also are studying the degradation pathway of urothelial endocytic vesicles, modified in their membrane lipid composition, by inducing the endocytosis of a pH – sensitive fluorescent probe. We found that almost all the probe endocytosed in control vesicles was acidified indicating the course to lysosomal degradative pathway. However, differential acidification kinetics dependent on the vesicular membrane lipid composition was observed, suggesting the regulatory role of lipids in proton translocation to the endocytic vesicles interior (unpublished data).

All the data mentioned, strongly support the existence of a degradation pathway via lysosome of the uroepithelial endocytic vesicles.

Another intracellular pathway of endocytic vesicles and their content was recently proposed by us [79]. We studied the release or leakage of endocytic vesicles content to the cytosol. We induced endocytosis of a fluorophorer – quencher complex, HPTS (hydroxypyrene-1,3,6-trisulfonic acid) and DPX (p-xylene-bis-pyridinium bromide) respectively and we determined the release or leakage mechanism and quantities of released material. For study the leakage phenomenon we used the requenching method developed by Wimley [80] and widely extented by Ladokhin [81]. The requenching method is based in a simple titration of HPTS fraction released from the endocytosed vesicle to determine the internal quenching of endocytic vesicles content. Basically the requenching method is comprised of simple fluorescence relations between total amount of fluorescence and extravesicular quenched fluorescence determined by the external addition of the "quencher" DPX. This external fluorescence titration allow us to experimentally determine two quenching parameters; Quenching total = Fluorescence / Fluorescence Maximum and Quenching outside = Fluorescence / Fluorescence Maximum outside. Deriving the fluorescence relations and plotting the two parameters determined experimentally we obtain a linear equation:

$$Q_{tot} = Q_{out} \cdot f_{out} + Q_{in} \cdot (1 - f_{out})$$

The slope of the linear function is the fraction of HPTS released f_{out} and from the interception we obtain the internal quenching Q_{in} [79, 80, 81].

The internal quenching dependence on the HPTS fraction released allow us to define two possible mechanism of leakage: a) the all-or-none mechanisms, where the internal quenching is independent of the HPTS fraction released and b) the graded mechanisms, with changes of the internal quenching dependent of the fluorescence fraction released. In the first case a population fraction of endocytic vesicles release all of their content and others do not; in the second case the fluorescence released from the endocytic vesicles is partial. If the release mechanism is All-or-None no further calculations from equation 4 is needed, however in case of Graded release mechanism a differential release of the fluorescent and its quencher is possible [79, 80] and we must use the extended theory of requenching method developed by Ladokhin [80]. In effect the differential release of the endocytosed material, HPTS-DPX pair, is determined by the α parameter, which represents the ratio of the rates of release of DPX and HPTS, k_{DPX}/k_{HPTS}. Now the internal quenching can be calculated by:

$$Q_{in} = [(1 + K_d \cdot [DPX]_0 \cdot (1 - f_{out})^{\alpha} \cdot (1 + K_s \cdot [DPX]_0 \cdot (1 - f_{out})^{\alpha})]^{-1}$$

where K_d and K_s are the dynamic and static quenching constants respectively obtained by the Stern-Volmer equation [82] and $[DPX]_0$ is the initial endocytosed DPX concentration.

Our results have shown not only the existence of leakage of the urothelial endocytic vesicles, but also the differential release mechanisms dependant of membrane lipid composition of the vesicles. In order to obtain changes of the vesicle membrane lipid composition we have used dietary strategies [7]. It results in three groups of vesicles, enriched respectively in linoleic acid, oleic acid and the third or control group. The control and linoleic acid rich vesicles showed a graded leakage mechanism, with preferential release of DPX, probably due to high content of phosphatydilserine of these membranes [8]. Due to the

anionic character of this phospholipid it may probably concentrates the cationic quencher DPX on membrane margins, and thus facilitating the DPX release.

In case of oleic acid vesicles, we couldn´t distinguish between the All – or – None leakage mechanism and the highly preferential release of HPTS, due to limitation of the requenching method. The oleic acid vesicles were found to contain a high content of phosphatidylethanolamine [8], and it is known that DOPE (dioleylphosphatidylethanolamine) is a non – bilayer – phase promoting lipid which favors the formation of inverted hexagonal (H_{II} or H_I) structures that confer membrane instability [83, 84]. This lipid properties may probably applied to the oleic acid vesicles where their high content of oleic acid may well be associated with the increased amount of the PE phospholipid found in this kind of membrane. This lipid interaction may well promote bilayer disruption with the appearance of non-bilayer and, consequently, a vesicular membrane destabilization leading to the non differential release of its luminal content.

This leakage pathway of the fluorophorer – quencher complex opens an interesting area of research, especially for studying the effect on the permeability barrier of the urinary bladder of others inflammatory metabolites or toxics provided by meals and eliminated through the urine.

7. UMBRELLA CELLS DIFFERENTIATION STAGE: CONSEQUENCES ON ENDOCYTOSIS

The cell culture is a powerful tool in Cell Biology, and great discoveries are related to this technique. The growing ability of the cultured cells allows us to reproduce their in vivo differentiation program under well defined and controlled in vitro conditions. Using this technique, several researchers have cultured mammalian urothelial cells, forming uroepithelium that mimic the phenotype of in vivo urothelium [91, 92]. In fact it was suggested that rat ureter urothelium is an accurate model for studying urothelial cells [24] and it was proposed that, in human, all the research obtained from ureter urothelial cultured cells can be applied to urinary bladder [87]. But exist opposite data between urothelial cultured cells that can [85, 88] and those that cannot [35, 89, 90] synthesize and assemble 2D crystals of urothelial plaques at the AUM. So, a controversy exists whether culture technique effectiveness could be related to the different laboratory protocols. However, urothelial cells differentiation can be determined by identifying differentiation markers such as uroplakins that are accumulated in highly differentiated umbrella cells [87, 91, 92]. In fact, using uroplakin and specific keratins as uroepithelium markers, Sun and col have demonstrated that the umbrella cells from bovine ureter shown less cytoplasm uroplakin staining that in bladder umbrella cells which correspond to a small number of endocytic vesicles determined by electron microscopy [79,80], and confirmed by inmunoblotting. We have already mentioned that umbrella cells synthesize keratins K8, K18 and K19 and traces of K5 and K6 [35, 36, 37], however bovine urothelial cultured cells also express K8, K18 and K19 and increase the synthesis of K5/K14 which are markers of keratinocyte basal cells [95, 98] and the markers of hyperproliferation K6/K16 [97]. Sun has interpreted those results as a tissue transformation. In effect, being originally the urothelium of a keratinocyte-type stratified squamous epithelium and later evolved to the normal urothelium, expressing only simple urothelial

keratins. However in hyperplastic conditions can return to the keratinocyte-like state [35, 83]. In fact, after urothelium destruction by cyclophosphamide, the cytokeratin K7 and uroplakin are the first to develop, but in a disorganized state, and relatively small vesicles are found. Later the K7 are gradually reorganized into a continuous network and uroplakins became organized into AUM, which will form the endocytic vesicles [98].

It has also been reported the differences in endocytosis related to the differentiation degree of umbrella cells.

Indeed, there is experimental data that demonstrate difference of the endocytosis process of umbrella cells in different maturation stages.

Kreft et al. had studied the non – stretched constitutive endocytosis in differential maturation stages of umbrella cells [22, 99]. They differentiate between the fluid phase endocytosis and the membrane bound endocytosis [100] being these two pathways dependant on the maturation stage of umbrella cells.

They found that in the highly differentiated umbrella cells (much uroplakin labellling) the constitutive endocytosis of fluid – phase endocytic marker was 32 % lower than that of the membrane – bound marker. In partially differentiated umbrella cells (less uroplakin labeling) this difference was even greater; the endocytosis of fluid – phase marker was 84 % lower than of the membrane – bound marker. They suggested that arrested fluid – phase endocytotic traffic is a necessary physiological adaptation of bladder urothelial cells, especially of the partially differentiated superficial cells with weak expression of uroplakins to maintain the permeability barrier [99].

It is important to remark that constitutive endocytosis is a characteristic of cultured umbrella cells and in vivo the endocytosis and exocytosis processes are related to the changes of hydrostatic pressure and changes of plasma membrane tension, described before, during the micturition cycle. The results obtained “in vitro”, where the absence of the micturition cycle will probably affect the cell membrane dynamic, are not strictly applicable to the cell function “in vivo”. Just to mention, the two dimensional crystalline array [87] does not develop in the urothelial cell culture which may imply a different uroplakin marker distribution in the surface of the plaque-deficient cultured cell and that may account for the difference between fluid phase endocytosis and membrane endocytosis.

8. CONCLUSION

In this chapter we discussed a very special endocytosis mechanism in normal urinary bladder umbrella cells due to changes of plasma membrane tension occurred during micturition cycle. In contrast to the widely known receptor mediated clathrin and caveolin dependant mechanism [100] where the endocytosis occurs in membrane domains and the endocytic vesicles formed are covered by the respective proteins mentioned, the umbrella cells endocytic vesicles are not covered neither clathrin nor caveolin. Moreover, the endocytosis in umbrella cells occurs by simple mechanical forces such as hydrostatic pressure and plasma membrane tension. This processes of vesicles edocytosis / exocytosis, resulting basically in plasma membrane traffic, is critically necessary because the plasma membrane of umbrella cells is a poor elastic structure and the increasing tension will rupture the membrane. Hicks developed in the 70s a model where the vesicles fusion to the plasma membrane during

filling phase and after bladder voiding the membrane is endocytosed restoring the initial pool. However new experimental data [72, 73, 74, 78, 79] indicated alternative pathways to the proposed by Hicks [24]. In this chapter we summarized the new pathways being basically four: the degradative – lysosomal, the recycling proposed by Hicks, the leakage to the cytosol and *de novo* sinthesis. Taking all the pathways together we developed a new model describing the traffic of the endocytosed vesicles, both membrane and fluid phase. The development of this new model opens new research areas especially in bladder pathology. The formation of urothelial plaques and apical plasma membrane traffic in umbrella cells are important to the formation and maintenance of the blood – urine permeability barrier. In many cases of disease this permeability barrier is altered. The consequents questions are where and when it happens. Gradually the researchers are responding to the questions. For example, it is known that ablation of UPIIIa and UPII genes in mouse abolishes the formation of uroplakin-delivering endocytic vesicles causing a compromised permeability barrier [101, 102]. And we recently described the permeability barrier dependence on lipid membrane composition [79].

After passing thirty years of the model proposition by Hicks, the traffic of the endocytic vesicles in bladder umbrella cells actually remains to be fully explored.

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Chapter 4

NEED TO ENDOCYTOSE? ABL TO HELP

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ABSTRACT

Cells use various mechanisms to internalize substances presented in their surroundings, with pinocytosis, phagocytosis, macropinocytosis, caveolae/raft-dependent endocytosis, and receptor-mediated clathrin-dependent endocytosis being the most extensively studied. Pinocytosis occurs independently of actin polymerization. In contrast, the importance of F-actin remodeling during internalization by the other mechanisms is well established. Similarly, the key role of membrane-proximal tyrosine kinases during internalization of small receptor ligands such as growth factors, or much larger bacteria, is undeniable. Surprisingly though, the molecular mechanisms linking tyrosine kinase activation and cytoskeletal reorganization pathways to endocytosis are not completely understood. cAbl is a member of the only family of tyrosine kinases known to date to have an actin-binding domain that allows them to interact with both globular and filamentous actin [1-7] and yet, our understanding of its role in endocytosis is still in its infancy. Herein, I review the evidence supporting a role for cAbl in the uptake of ligated receptors, apoptotic cells, bacteria and viruses. Specifically, I describe and discuss cAbl-dependent pathways involved in 1) clathrin-mediated endocytosis of two different receptors (EGFR and BCR), 2) caveolin-dependent endocytosis of viruses, and 3) macropinocytosis of bacteria and apoptotic cells, highlighting the overlap and differences between them. Finally, I discuss the potential significance of having specific endocytic mechanisms adapted to given conditions, as well as the significance of cAbl's role in endocytosis linked to physiological signal transduction and pathological conditions such as cancer and fibrosis.

INTRODUCTION

The Abl family of non-receptor tyrosine kinases is comprised of cAbl, Arg[1] (Abl-related gene) and Abl-derived oncoproteins such as vAbl and Bcr-Abl. Because of its involvement in 95% of cases of chronic myelogenous leukemia (CML), Bcr-Abl has been extensively studied and characterization of STI571 (also known as imatinib, Gleevec®, Glivec® and CGP57148B) as an inhibitor of Bcr-Abl kinase activity has led to its widespread use in the treatment of CML patients. On the other hand, STI571 has provided a critical tool to investigate the role of endogenous cAbl in physiologic cell signaling. We ought to be cautious when interpreting data obtained with STI571-treated cells because the drug has been shown to inhibit all members of the Abl family of tyrosine kinases, as well as PDGF receptor, c-kit and c-fms [reviewed in 8-14]. However, the combination of pharmacological and genetic studies has revealed valuable information. There is now substantial evidence to back a role for cAbl in cytoskeletal reorganization leading to changes in cell shape and migration [1, 15-19]. Similarly, as I discuss in details below, there is mounting evidence to support a role for cAbl in actin-dependent macropinocytosis. In contrast, very little is known about the role of cAbl in ligand-induced receptor-mediated endocytosis.

CONSTITUTIVELY ACTIVE cABL INHIBITS LIGAND-INDUCED EGFR ENDOCYTOSIS

In 2006, Tanos and Pendergast [20] used fibroblast-like COS7 cells to over-express two different forms of constitutively active cAbl and showed that these mutants reduced (but did not block) endocytosis of EGF-EGFR complexes, compared to cells transfected with a control vector. This suggested that constitutively active cAbl negatively regulates ligand-induced receptor internalization. In contrast, COS7 cells transfected with a kinase-dead (K290M) mutant of cAbl internalized EGF-EGFR complexes as efficiently as the mock-transfected cells, indicating that the kinase activity of cAbl is required for the negative regulatory effect to occur. They also showed that over-expression of dominant-negative dynamin K44A in COS7 cells had a similar inhibitory effect on internalization of the complexes, which they interpreted to suggest that constitutively active cAbl inhibits receptor-mediated clathrin-dependent endocytosis in this cell system [20]. It is indeed well established that dynamin, along with clathrin, AP2, epsin, cCbl and many other proteins, is a marker of clathrin-mediated receptor internalization [reviewed in 21, 22]. However, there is also some evidence that dynamin participates in caveolin-dependent endocytosis [21]. In order to discriminate between these two potential mechanisms, the authors looked at the distribution of cCbl upon EGF stimulation. cCbl is a ubiquitin ligase that has been shown to mark ligated receptors for

[1] Arg, Abl-related gene; Abi-1, Abl-interacting protein 1; BCR, B cell antigen receptor; CagA, cytotoxin-associated gene A; CAR, Coxsackievirus and adenovirus receptor; CML, chronic myelogenous leukemia; CVB, group B Coxsackieviruses; DAF, dacay-accelerating factor; EGF, epidermal growth factor; EGFR, EGF receptor; Ig, immunoglobulin; MEFs, mouse embryonic fibroblasts; MHCII, major histo-compatibility complex class II; NRTK, non-receptor tyrosine kinase; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; RTK, receptor tyrosine kinase; TCR, T cell antigen receptor; T3SS, type III secretion system; T4SS, type IV secretion system; wt, wild-type.

internalization and degradation or recycling, depending on the level of uniquitylation, in various systems [23-29]. Their immunoprecipitation data revealed that cCbl associated with EGFR in EGF-stimulated wt cells (compared to untreated cells) and that there was no such EGF-induced association in cells over-expressing constitutively active cAbl. It will be important to confirm the role of cCbl by demonstrating, for example, that in COS7 cells cCbl knockdown has a similar effect on EGF-EGFR internalization as the dominant negative dynamin. Nevertheless, ligand-induced endocytosis of EGFR does appear to proceed via the canonical clathrin-dependent pathway and constitutively active cAbl appears to inhibit it.

ENDOGENOUS CABL PROMOTES LIGAND-INDUCED BCR ENDOCYTOSIS

Interestingly, our laboratory later reported that in the DT40 B cell line, endogenous cAbl positively regulates ligand-induced endocytosis of B cell antigen receptor (BCR) [30]. We used cells in which cAbl had been knocked-out (DT40cAbl$^{-/-}$ cells) and showed that anti-Ig-induced BCR endocytosis was significantly delayed (but not ablated) in the absence of cAbl expression, compared to wild-type (wt) cells. Likewise, we showed that in the absence of cAbl activity (parental DT40 cells treated with STI571), anti-Ig-induced BCR endocytosis was remarkably delayed but not abolished, compared to untreated wt DT40 cells [30]. The effect observed in cAbl knockout cells was specific, as it was reversed by reconstitution with cDNA coding for wt cAbl but not kinase-dead (K290R) cAbl, thereby establishing the requirement for cAbl kinase activity. We also determined that over-expression of wt cAbl enhances anti-Ig-induced BCR endocytosis, which is consistent with a positive regulatory role of cAbl in receptor-mediated endocytosis. Moreover, our data implicating cCbl in Ig-induced BCR endocytosis [31] suggest that cAbl is required for optimal endocytosis of Ig-BCR complexes via the clathrin-dependent route (see above). Specifically, we demonstrated that cells in which cCbl expression had been knocked-out (DT40cCbl$^{-/-}$) exhibited an endocytic defect that paralleled that observed in cAbl knockout cells, that the defect was reversed by reconstitution with cCbl and thus specific, and that regulation of key signaling molecules (CrkII and Rac) was similarly affected in both DT40cCbl$^{-/-}$ and DT40cAbl$^{-/-}$ cells [30, 31].

At first glance, it may seem perplexing that cAbl could have opposing roles in clathrin-mediated endocytosis in different cell types. However, it is also possible that different receptors are regulated differently. Moreover, we cannot exclude the possibility that anti-Ig-BCR complexes may be processed differently because anti-Ig does not represent a natural ligand for BCR, as opposed to EGF for EGFR. Due to their role in antigen presentation, B cells represent professional endocytic cells compared to fibroblasts, and might thus utilize distinct regulatory mechanisms. Antigens bound to BCRs must be efficiently internalized and proteolytically processed before antigenic fragments can traffic back to the cell surface in association with MHCII molecules, where they are presented to cognate T cells to induce an immune response. On the other hand, EGF may instead function in endosomal signaling and thus represent a different type of cargo that initially transits along the same clathrin-dependent endocytic route but is differentially regulated by cAbl. It is also possible that receptors with intrinsic tyrosine kinase activity, such as EGFR, and those without (e.g. BCR, TCR) require a different contribution from cAbl. Finally, we must also consider the

possibility that the conformation of constitutively active cAbl mutants is different from that of endogenous wt cAbl, which would in turn likely affect the way it interacts with other proteins, as well as the overall regulatory mechanism. As I will discuss later, alterations in the association of cAbl with other proteins could lead to the development of pathological conditions such as cancer.

Considering this apparent contradiction about the role of cAbl in clathrin-dependent receptor-mediated endocytosis, future studies should include a direct comparison of wt and constitutively active cAbl in the same cell system. Ideally, the 3D structure of the wt and active mutant proteins in question should be determined and compared to uncover a potential structural effect that could be due to amino acid replacement. If there is such a structural effect, it could certainly account for the difference between the positive and negative effects reported for wt and constitutively active cAbl, respectively. Consistent with this line of thought, the cDNAs used by Tanos and Pendergast [20] contained activating proline mutations in the SH3 or SH2 domain of cAbl. Proline residues in the context of SH3 domains are well-known effectors of protein-protein interactions [reviewed in 32-36]. Therefore, such mutations would likely interfere with the ability of cAbl to associate with its physiologic partners and, consequently, alter its overall function. Similarly, mutations in the SH2 domain may impair cAbl's ability to interact with its usual tyrosine phosphorylated binding partners, which again would be expected to affect its function. It would also be informative to compare internalization of anti-Ig-BCR, antigen-BCR, and growth factor (GF)-GF receptor (GFR) complexes in B cells, as well as endocytosis of GF-GFR in professional vs non-professional endocytic cell types. Last but not least, analysis of the post-internalization fate of the different ligands and their receptors –i.e. degradation vs recycling vs endosomal signaling- needs to be performed to help resolve these issues.

CAVEOLAE-DEPENDENT ENDOCYTOSIS IS POSITIVELY REGULATED BY CABL

In the case of clathrin-dependent endocytosis[2], it is widely accepted that the binding of ligands (e.g. growth factors, cytokines) to their cognate receptor initiates the signaling cascade that eventually culminates in internalization of the ligand-receptor complexes. Viruses must also bind cellular receptors in order to enter host cells and access their cellular machinery. Once they do, they can replicate and spread to infect additional cells. As part of their work to elucidate the mechanisms used by Group B coxsackieviruses (CVB) to cross intestinal or respiratory epithelium and initiate infection, Coyne and Bergelson showed that cAbl plays a key role in the early steps of infection [37]. Coxsackievirus and adenovirus receptor (CAR) is the typical receptor for CVB, as well as other Coxsackieviruses and adenoviruses. CAR expression is restricted to the basolateral side of polarized host epithelial cells and is absent from their apical surface. Therefore, CVB arriving from the luminal/apical side cannot infect epithelial cells via CAR and must use an alternative route. DAF (decay-

[2] Although there are reports of bacteria entering cells via the canonical clathrin-dependent pathway [22], the involvement of cAbl in this process has not been explored and, therefore, these cases will not be discussed herein.

accelerating factor) has been shown to bind CVB and, unlike CAR, DAF is abundant on the apical surface of epithelial cells, thereby providing the so-called alternative entry route. How DAF works though remains poorly defined. Coyne and Bergelson [37] showed that binding of CVB to DAF triggers intracellular activation of cAbl, which in turn initiates signaling events that ultimately lead to the redistribution of the virus to tight junctions where they can bind CAR and induce internalization via a caveolin-dependent (clathrin-independent) pathway. Specifically, they used immunofluorescence staining to show that exposure of Caco-2 monolayers to CVB induced clustering and redistribution of DAF from the apical cell surface to the tight junctions, and that the clusters co-localized with F-actin. Given that cAbl mediates F-actin remodeling in response to growth factors and extracellular matrix signals in other cell systems (reviewed by Woodring et al. [4]), they then tested their hypothesis that cAbl was responsible for cytoskeletal remodeling in response to CVB-DAF binding. Their results indicated that active endogenous cAbl (phosphorylated on Tyr412) co-localized with DAF and F-actin in cells incubated with CVB, as compared to untreated cells, and that the kinetics of cAbl activation coincided with the kinetics of DAF clustering, suggesting that cAbl played a role in the mechanisms underlying CVB infection of epithelial cells. Consistent with this conclusion, the authors showed that STI571 prevented CVB-induced relocation of DAF and completely prevented infection. Furthermore, STI571 abolished the activation of Rac, a small GTPase that acts downstream of cAbl in numerous cell systems [30, 38-43]. These results are certainly indicative of a role for cAbl in the DAF-mediated infection of Caco-2 (epithelial cells derived from a colorectal adenocarcinoma) by CVB. However, because the effects of STI571 are not exclusively limited to members of the Abl family, the next step should include experiments that –for example- make use of RNAi technologies to confirm the specific involvement of cAbl, as opposed to Arg, PDGFR (see below), or other targets of STI571.

PHAGOCYTOSIS VS MACROPINOCYTOSIS

Phagocytosis also relies on well-defined receptors and can be used to remove apoptotic cells, necrotic cells, as well as microorganisms such as bacteria and viruses [reviewed in 44]. During embryogenesis, neighboring cells appear to be responsible for the phagocytosis of dead cells and it is only in adult tissues that professional phagocytes (such as neutrophils, macrophages and dendritic cells) become involved [45]. Phagocytes possess pattern-recognition receptors (e.g. scavenger receptors, the mannose receptor, Fc receptors and complement receptors) that recognize molecular patterns found on dying/dead cells and pathogens. As far as bacteria uptake is concerned, these receptors can induce signaling mechanisms that cooperatively recognize, capture, and internalize bacteria in order to kill and degrade them. Depending on the receptors involved, professional phagocytes can exploit distinct molecular and morphological processes to do so [46]. Thus, for example, Fc receptor-dependent internalization of IgG-opsonized pathogens is characterized by membrane extension and production of pro-inflammatory mediators whereas engulfment of complement-opsonized bacteria (mediated by complement receptors) is not; complement-coated bacteria simply appear to “sink” into the responding phagocyte without release of inflammatory molecules [46]. Importantly, Toll-like receptors (TLR) are implicated in the signaling cascade

that leads to activation of inflammatory responses during phagocytosis but not in the internalization process itself [46, 47]. Regardless of this "variation on a theme", the encounter between bacteria and phagocytes lies at the core of innate immunity [48, 49]. Additionally, in vertebrates, professional phagocytes, also known as antigen presenting cells (APC), can use bacterial degradation products they generate, load them onto MHC molecules and present the resulting complexes to helper T lymphocytes which then induce adaptive immunity to boost the overall immune response [45, 47-49].

In contrast to phagocytosis and clathrin-dependent endocytosis, the receptors involved in macropinocytosis (exploited by invasive bacteria to enter non-professional phagocytic cells such as epithelial cells) are not always well defined. After using diverse strategies to escape the killer embrace with professional phagocytes [46], invasive bacteria force their way into non-phagocytic cells of the intestinal epithelium (for example) that are not equipped to eradicate them and thus become infected [46, 49]. Two groups of invasive bacteria have been described based on their mechanism of entry into non-phagocytic cells: the zipper and trigger types [49]. In Gram-positive *Listeria*, the zipper mechanism involves interactions of In1A and In1B proteins on the bacterial surface with cellular E-cadherin and Met, respectively. These molecular interactions activate a signaling cascade that induces cytoskeletal reorganization and formation of membrane extensions that zip around bacteria to engulf them. On the other hand, in Gram-negative bacteria such as *Salmonella* and *Shigella*, contact with the host cells triggers remodeling of the actin cytoskeleton, membrane ruffling, pseudopod formation around the bacteria, and eventually their uptake, by injecting the so-called type III secretion system (T3SS) [reviewed in 50-58]. Following contact between an epithelial cell and *Salmonella* or *Shigella*, the T3SS inserts proteins into the plasma membrane of the host cell as part of a pore complex. This pore then allows bacteria to deliver additional effecter proteins into host cells, prompting the cascade of events described above.

cAbl Mediates Macropinocytosis in Gram-Negative Bacteria

Shigella uptake has been shown to require Cdc42 and Rac, both of which are members of the Rho family of small GTPases and key regulators of actin dynamics. Given the evidence that cAbl regulates Rac activity in other cellular pathways/systems [38-42], including anti-Ig-induced endocytosis of BCR [30], Burton and colleagues [43] investigated the role of cAbl in *Shigella* internalization. They used mouse embryonic fibroblasts (MEFs) that were cAbl/Arg-null, as well as wt fibroblasts that were pre-treated or not with STI571, incubated them with S. *flexneri* and determined the extent of macropinocytosis in each condition. They showed that Arg and/or cAbl are required for infection of non-phagocytic cells by invasive *Shigella*. Their double knockout system did not allow them to ascertain whether cAbl played a role at all in this process as Arg could have been solely responsible for the observed effects [43]. Therefore, additional studies with cAbl and Arg single knockout MEFs are required to address this key point. Nonetheless, their results indicated that genetic ablation of Abl/Arg expression or pharmacologic inhibition of their kinase activity decreases the extent of bacteria uptake. Furthermore, using HeLa and NIH-3T3 cells, the authors confirmed the involvement of Crk (a cytoskeletal adaptor protein that is devoid of enzymatic activity and is the best

characterized substrate of cAbl to date [59]) and Rac (a small GTPase known to regulate actin reorganization) downstream of cAbl/Arg in this endocytic pathway. Interestingly, these results are similar to what we reported in our investigation of anti-Ig-induced BCR internalization (see above) [30]. Perhaps even more revealing though is the fact that their results indicate a positive effect of cAbl/Arg on macropinocytosis, consistent with the positive effect of cAbl on BCR endocytosis that we reported [30] but opposite to the negative effect of constitutively active Abl on EGFR internalization reported by Tanos and Pendergast [20] (see Figure 1). This emphasizes the need to 1) evaluate the effects of wt and constitutively active cAbl in this system and 2) compare the 3D-structure of wt and constitutively active forms of cAbl. It also reinforces how critical it will be to look into the trafficking of ligand-receptor complexes and engulfed bacteria to refine our understanding of cAbl's role in various endocytic processes.

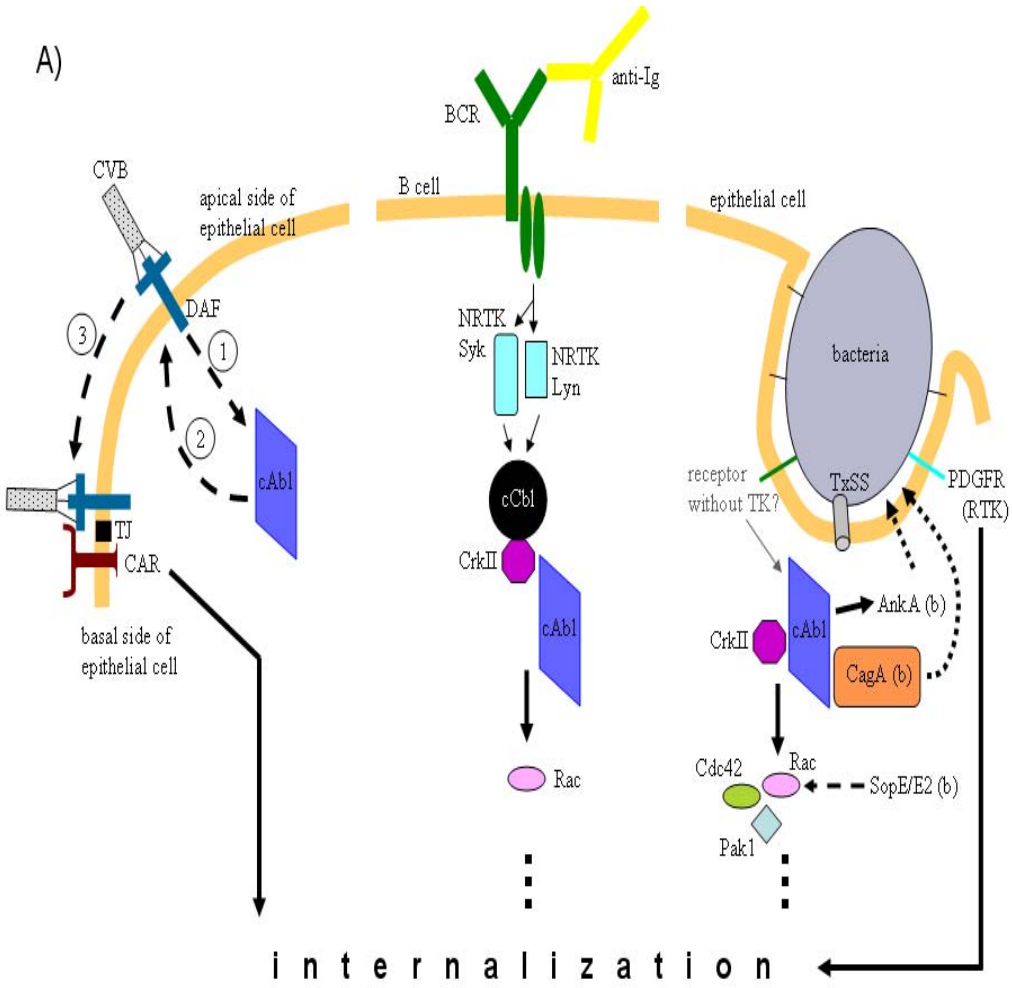

Figure 1. (Continued)

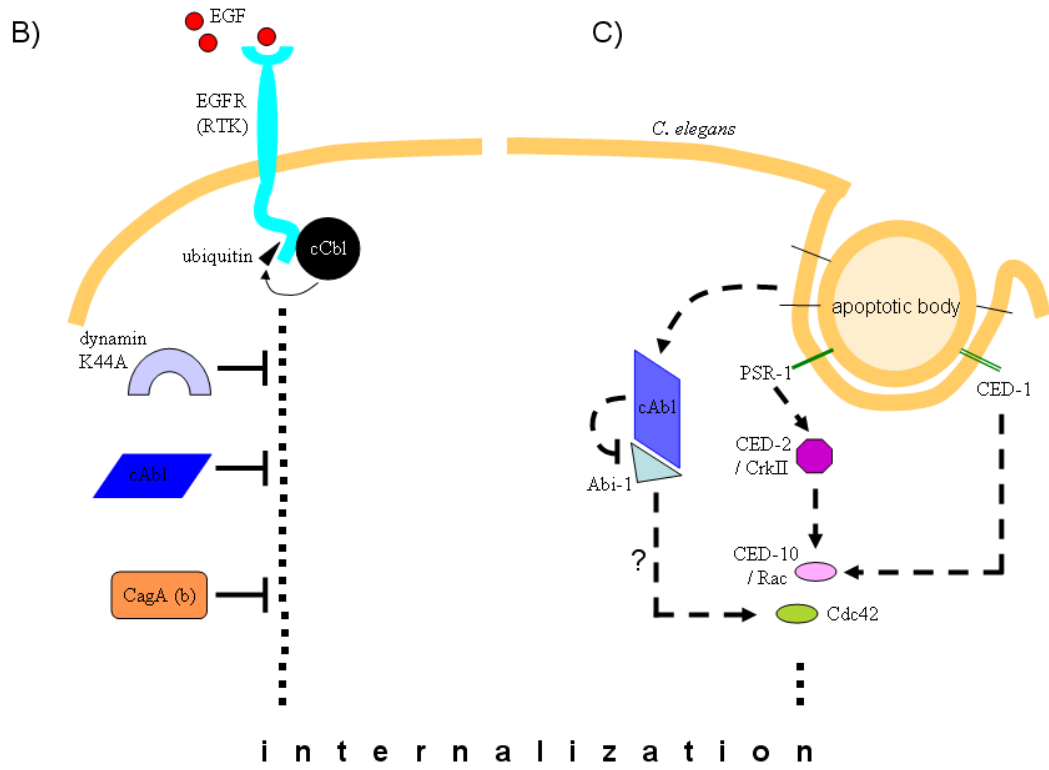

Figure 1. A) Endogenous cAbl plays a positive role in caveolin-dependent internalization of viruses (left), ligand-induced endocytosis of BCR (center), and macropinocytosis of bacteria (right). B) Over-expression of constitutively active cAbl inhibits ligand-induced of EGFR. C) The *C. elegans* homolog of cAbl, ABL-1, inhibits macropinocytosis of apoptotic cells. The literature indicates that over-expression of constitutively active cAbl (dark shade of blue) inhibits ligand-induced endocytosis of EGFR, a receptor tyrosine kinase (RTK), while endogenous wt cAbl (lighter shade of blue) promotes ligand-induced endocytosis of BCR, a receptor that is devoid of enzymatic activity and relies on cytosolic tyrosine kinases (also known as non-receptor tyrosine kinases, or NRTK) to signal. Abbreviations: TJ, tight junction; TK, tyrosine kinase; TxSS, T3SS or T4SS.

As another typical trigger type of bacteria, Salmonella also uses the T3SS apparatus [50-58] to promote Rac activation and the cytoskeletal reorganization necessary for the formation of actin-rich membrane protrusions that eventually engulf the attached bacteria. Considering that RNAi-mediated depletion of Abi-1 (Abl-interacting protein 1, also known as Abl-interactor 1, or Abelson-interacting protein 1) resulted in reduced entry of Salmonella into cells [60], and that Abi-1 is often associated with members of the Abl family, Ly and Casanova [61] hypothesized that Abl kinases also contribute to the mechanisms underlying *S. enterica* (serovar Typhimurium) invasion. They demonstrated that the extent of *S. enterica* infection was 70% lower in MEFs lacking both cAbl and Arg proteins, compared to wt MEFs. They also presented evidence that cAbl is enriched at sites of bacterial entry, and that siRNA-mediated depletion of cAbl reduces bacteria uptake. Taken together, these data suggest that cAbl plays a positive regulatory role in S. *enterica* internalization. Consistent with these findings, the authors demonstrated that HeLa and MDCK cells pre-treated with STI571 both had a significantly lower index of internalization, compared to untreated cells.

This is significant as these two epithelial cell lines are likely more representative of the intestinal epithelial cells normally infected *in vivo* by *S. enterica* than MEFs are. It is also worth noting that, even though the positive effect of cAbl on *S. enterica* uptake is consistent with the positive regulatory role of cAbl in anti-Ig-induced BCR endocytosis [30] and macropinocytosis of *S. flexneri* [43], STI571 had no effect on Rac activity during the engulfment of *S. enterica*. This particular piece of data contrasts with the other two reports. This may seem perplexing at first but other laboratories have shown that among the effecter proteins that *Salmonella* injects into host cells via the T3SS apparatus are SopE/E2. Importantly, these proteins can activate Rac1 directly [62, 63] and do not require cAbl, which explains why STI571 was ineffective in this system. It thus seems that different types of trigger bacteria may have evolved to use different versions of the same endocytic pathway.

Salmonella and *Shigella* both use the T3SS to initiate infection of host cells. However, at least six different specialized secretion systems have been identified and characterized among Gram-negative bacteria [64-66]. The T3SS acts like a molecular syringe through which bacteria can inject proteins into eukaryotic cells. The T4SS is more related to the conjugation system and allows bacteria to inject DNA and/or proteins into host cells [67-69]. In 2007, Lin et al. [70] published data suggesting that cAbl plays a role in the T4SS-mediated infection of HL-60 cells by *Anaplasma phagocytophilum*. Indeed, they showed that pre-treatment of the cells with STI571 practically abolished uptake of bacteria by those cells, compared to vehicle-treated cells. At the molecular level, they showed that AnkA, a bacterial protein, is introduced into host cells via the T4SS, and that this is required in order for *A. phagocytophilum* to infect HL-60 cells. Once inside host cells, AnkA associates with Abi-1 and is phosphorylated by cAbl. Furthermore, they demonstrated that siRNA-mediated depletion of cAbl proteins significantly reduced tyrosine phosphorylation of AnkA [70]. From these results, the authors concluded that cAbl plays a key role in the engulfment of *A. phagocytophilum*. These findings are very interesting and informative, and they are in agreement with the data reported by other groups who studied the role of endogenous wt cAbl in endocytosis, and macropinocytosis in vertebrates [30, 37, 43, 61, 70-72]. However, in future studies, it will be important to establish that siRNA-mediated depletion of cAbl does in fact inhibit macropinocytosis in this system to confirm the proposed correlation between cAbl-mediated tyrosine phosphorylation of bacterial AnkA and infection of host cells.

In 2008, Engel's laboratory published two papers in which they used RNAi technology to try to elucidate the pathway(s) usurped by *Chlamydia trachomatis* [71] and *Pseudomonas aeruginosa* [72] during host cell infection. In their investigation of Gram-negative *Pseudomonas aeruginosa*, they inactivated 80 genes known to be involved in cytoskeletal remodeling and determined their effect on macropinocytosis [72]. Among the genes investigated, they identified cAbl and some of its known downstream signaling effecters (Crk, Rac, Cdc42 and PAK) as mediators of the T3SS-mediated pathogen entry into murine 3T3 fibroblasts or human HeLa epithelial cells. Specifically, they showed that bacteria uptake was significantly decreased (about 65%) in $cAbl^{-/-}Arg^{-/-}$ double knockout 3T3 cells, compared to wt 3T3 cells. They also showed that STI571 inhibited invasion of HeLa cells by 50 to 75%, although the doses required for this inhibitory effect (30 and 50 μM) [72] are significantly higher than what is typically used (10 μM) in the literature. In the system in question, 10 μM STI571 had no significant effect. Since STI571 can affect Abl kinases, PDGFR, c-kit and c-fms (see above), these findings in themselves are not sufficient to conclude that cAbl is

involved in this process. However, specific depletion of cAbl proteins using RNAi also reduced the level of invasion of HeLa cells by the pathogen by over 50% [68], suggesting that cAbl is indeed implicated in the mechanisms leading to *P. aeruginosa* infection of non-professional phagocytes. Considering that in both experiments cell invasion was not entirely blocked –despite the complete absence of cAbl protein- it appears that cAbl promotes but is not essential for bacteria uptake, which is similar to what we reported in our studies of anti-Ig-induced endocytosis of BCR [30].

When siRNA-mediated depletion of Crk was performed in this system, invasion of HeLa cells by *P. aeruginosa* dropped by ~40% (compared to cells treated with control siRNA), which correlated with a reduction of CrkII protein levels by ~50% [72]. Similarly, they observed a 40-50% inhibition of invasion following a comparable reduction in the levels of Rac protein via siRNA. Finally, they showed that a modest reduction in Pak1 protein levels resulted in an equivalent decrease (~35%) in invasion of HeLa cells by the pathogen. It is also important to mention that the authors found that over-expression of Y221FCrkII (a mutant form of CrkII that cannot be phosphorylated by cAbl) caused a significant (~40%) decline in the rate of HeLa cell invasion. This is intriguing because Y221FCrkII is known not to have dominant-negative properties, compared to other CrkII mutants [59]. Nevertheless, the results –taken together- point to a positive role for wt cAbl, CrkII, Rac and Pak1 in the signaling cascade leading to internalization of *P. aeruginosa* and cell infection.

In future experiments, it will be very interesting to see whether a complete ablation of CrkII protein results in a complete inhibition of cell invasion by *P. aeruginosa* (or other pathogens). We previously suggested that CrkII acts as a linchpin between a cAbl-dependent pathway leading to remodeling of the actin cytoskeleton and a cCbl-dependent pathway responsible for the ubiquitylation of proteins destined to degradation after internalization [30, 31]. In our proposed model, CrkII connects these two key pathways to ensure optimal endocytosis of ligated receptors. However, the cell system that was used in our investigation of ligand-induced endocytosis of BCR molecules is not yet amenable to siRNA technologies. Despite the numerous advantages of the DT40 cell lines, such as a high rate of homologous recombination and the low level of protein redundancy, our multiple attempts to knockdown CrkII in the chicken cells expression failed. Therefore, our hypothesis remains to be tested.

MACROPINOCYTOSIS OF GRAM-INDETERMINATE *CHLAMYDIA TROCHOMATIS* DEPENDS ON CABL

Elwell et al. [71] also used the same cell systems (HeLa and NIH3T3) to study the mechanism involved in macropinocytosis of *Chlamydia trachomatis*. Unlike *P. aeruginosa* and other Gram-negative bacteria, *C. trachomatis* do not have a peptidoglycan layer [73] and are thus considered Gram-indeterminate. In this case, the bacterial ligands and host cell receptors involved have yet to be defined but induction of microvilli-like structures over a large surface of the host cells has been observed and determined to be actin-dependent. Similarly, tyrosine phosphorylated proteins have been shown to accumulate at the site of bacterial entry but few have been identified. Elwell et al. used a library of over 7,200 siRNA

to identify 226 host genes that were implicated in the process [71]. They showed that cAbl, as well as Vav2, WAVE2 and cortactin[3] (all three of which have been shown to be important for Rac-mediated rearrangement of F-actin in various other systems) were among those genes. Interestingly, PDGF receptor (PDGFR) was also among those genes. This is an important piece of information because, as mentioned above, the drug commonly used to study cAbl-dependent processes, STI571, is well known to inhibit all members of the Abl family, as well as PDGFR, c-kit and c-fms [8-14]. Specifically, the authors demonstrated that after siRNA-mediated depletion of PDGFRβ protein levels by 52%, the percentage of HeLa cell-associated bacteria was similarly reduced by ~50% [71]. In contrast, siRNA-mediated depletion of cAbl with an efficiency of 60% did not have any significant effect on the extent of *C. trochomatis* binding to the cells, which is consistent with their data from similar experiments with *P. aeruginosa* [72] and suggests that PDGFR but not cAbl is important for binding bacteria prior to infection. They also showed that pre-treatment of HeLa or NIH3T3 cells with STI571 –which inhibits both cAbl and PDGFR- decreases the internalization efficiency by ~40% whereas AG1295 (a specific inhibitor of PDGFR) had no effect. Furthermore, their results indicated that cAbl/Arg-double knockout cells were as efficient in engulfing bacteria as the parental cells [71]. In this double knockout cells, however, AG1295 inhibited macropinocytosis by ~40%, which suggested that cAbl and PDGFR function redundantly in the mechanism underlying *C. trochomatis* entry into cells of the non-professional phagocytic type. These data also suggest that PDGFRβ can act as a receptor for bacteria binding, thereby initiating internalization, because it is a receptor tyrosine kinase with intrinsic tyrosine kinase activity. On the other hand, cAbl being a non-receptor cytosolic tyrosine kinase, it likely relies on a distinct receptor to bind *C. trochomatis* and induce internalization.

To confirm their results, the authors treated cells that had been transfected with cAbl siRNA or control siRNA with STI571 or AG1295, and compared their capacity to internalize bacteria. They observed that unless both cAbl and PDGFR were prevented from acting, internalization indeed proceeded normally [71]. Finally, they used a STI571-resistant mutant form of cAbl (Abl-T351) to support their conclusion by showing that over-expression of this mutant was sufficient to bypass STI571 and restore the level of internalization back to that found in wt untreated cells. One of the questions that remain open pertains to the overall level of inhibition attained in this set of experiments. The fact that the maximum inhibition was ~40%, regardless of whether cAbl, PDGFR, or both were inhibited, indeed suggests that other receptors and/or mechanisms are at play during macropinocytosis of *C. trichomatis*. This question warrants additional experiments as it might help determine whether –in this system- cAbl is required to ensure signaling initiated by a receptor that is itself devoid of intrinsic tyrosine kinase activity. And although it is doubtful that PDGFR could play a similar direct role in anti-Ig-induced [30, 31] or EGF-induced [14] receptor endocytosis (because of the nature of the ligands themselves), it will certainly be informative to study the role of PDGFR in other systems of host cell infection by bacteria.

[3] The phosphorylation status of Vav2, WAVE2 and cortactin was assessed using immunochemical techniques but was not directly correlated with endocytosis data.

THE *C. ELEGANS* HOMOLOG OF CABL, ABL-1, DOWN-REGULATES ENGULFMENT OF APOPTOTIC CELLS

As mentioned earlier, removal of apoptotic cells by neighboring cells or phagocytes is essential for normal development and tissue remodeling. In *Caenorhabditis elegans* (*C. elegans*), two partially redundant pathways assume this task during gonadogenesis: one signals through CrkII/CED-2 and Rac1/CED-10 to induce cytoskeletal rearrangement while the other works via CED-1 (a receptor homologous to the mammalian EGF-like receptor MEGF10) and seems to cause the plasma membrane to extend around the apoptotic cell [74]. Because cAbl is known to regulate CrkII and Rac in different systems [30, 38-43], Hurwitz et al. [75] investigated the hypothesis that the homolog of cAbl in *C. elegans*, ABL-1, regulates CrkII/CED-2-dependent uptake of apoptotic cells generated during the gonadogenesis process. Specifically, they counted the number of apoptotic cells/bodies left not internalized after a specific time in wt worms, as well as worms carrying various gene-inactivating (null) mutations. They showed that wt *C. elegans* internalized all the apoptotic cells generated and that *abl-1* mutations (n1963 or ok171) alone had no obvious effect on this phenotype [75]; the *abl-1* mutant worms also internalized all the apoptotic corpses generated. However, mutation of *ced-1* or *ced-2* caused accumulation of non-internalized corpses, reducing engulfment without blocking it completely, consistent with the two-branch model summarized above [75]. Interestingly, mutation of both *abl-1* and *ced-1* partially restored uptake of apoptotic bodies. Likewise, mutation of both *abl-1* and *ced-2* partially restored engulfment of apoptotic bodies. These results suggest that ABL-1 down-regulates the internalization process (Figure 1C). Moreover, the fact that ABL-1 inhibited the uptake of apoptotic cells in the absence of CrkII/CED-2 or CED-1 suggests that the effect of ABL-1 on engulfment does not require CrkII/CED-2 or CED-1. In contrast, mutation of *abl-1* in the *ced-1*/*ced-2* double mutant had no effect, suggesting that in *C. elegans* the inhibition of macropinocytosis by ABL-1 may require an effecter acting downstream of CED-1 and CrkII/CED-2. The authors hypothesized that Rac/CED-10 may be that effecter and thus determined the rate of internalization in *ced-10* mutant. They showed that engulfment was decreased in *ced-10* (to a similar extent as the ced-1 and ced-2 single mutants) and that ablating ABL-1 expression partially restored the defect. Given that the uptake of apoptotic cells was abolished in the ced-1/ced-2 double mutant, it may seem surprising that it was not completely ablated in the absence of CED-10/Rac connecting the CED-1 and CrkII/CED-2 pathways [75]. However, it is possible that CDC42ce, the CDC42 homolog known to be expressed in *C. elegans* compensates for the absence of CED-10/Rac. Indeed, Cdc42 could play a role in the uptake of apoptotic cells, similar to its role in the macropinocytosis of S. flexneri (see above and Figure 1A, 1C).

The fact that the absence of ABL-1 alone did not cause any phenotype is somewhat intriguing and warrants further investigation. For example, it would be worth repeating the engulfment experiments after setting the maximum level of uptake in wt animals at 50%, instead of 100% which might represent supra-optimal conditions. If ABL-1 turns out not to be essential but to have a kinetic effect –similar to what we observed in B cells- the new set-up might then reveal a phenotype in the *abl-1* null animals. Nevertheless, these results are interesting, especially when considering that CED-1, a receptor thought to mediate the uptake of apoptotic cells in *C. elegans*, is homologous to the mammalian EGF-like receptor MEGF10 and thus related to EGFR. As discussed earlier, ligand-induced endocytosis of

EGFR has also been shown to be negatively regulated by cAbl. However, in contrast to EGFR, CED-1 does not appear to have intrinsic tyrosine kinase activity and is thus not a RTK. Therefore, the presence or absence of intrinsic tyrosine kinase activity may not be sufficient to explain why BCR and EGFR are differentially regulated by cAbl (see above and Figure 1).

A POSITIVE EFFECT OF CABL ON *H. PYLORI* UPTAKE LEADS TO A NEGATIVE FFECT OF CABL ON EGFR ENDOCYTOSIS

Helicobacter pylori is another pathogen that can infect non-phagocytic epithelial cells. In fact, it is one of the most successful type of bacteria, colonizing roughly 50% of the human population worldwide. Like many other Gram-negative bacteria, *H. pylori* depends on the T4SS [67-69] to inject the effecter protein CagA (cytotoxin-associated gene A), among others, and ultimately enter host cells. Once into the target cells, CagA rapidly becomes phosphorylated by the tyrosine kinase Src. However, unlike Src activation that is transient and short-lived, tyrosine phosphorylation of CagA is sustained in cases of persistent *H. pylori* infection. This has prompted researchers to look for another kinase that could mediate the second permanent phosphorylation event. Subsequently, two groups identified cAbl as a key player in the signaling cascade leading to sustained phosphorylation of CagA and *H. pylori* infection [77, 77]. Poppe et al. [77] showed that cAbl directly interacts with CagA upon infection, and that inhibition of cAbl activity with STI571 or depletion of the protein with specific shRNA abrogates CagA phosphorylation. They also demonstrated that inhibitors of EGFR (PP3 and AG1478) and PDGFR (AG1295) had no effect on tyrosine phosphorylation of CagA. In contrast, the Src inhibitor PP1 significantly reduced the level of phosphorylated CagA, consistent with its role in mediating the first, transient event.

The data obtained by Poppe et al. in the presence of the PDGFR inhibitor may appear intriguing considering the report by Etwell et al. [71] that indicated a role for both cAbl and PDGFR in *C. trachomatis* infection of epithelial cells or fibroblasts (see above). However, we must keep in mind that Poppe et al. did not investigate the entry step per se, but rather the motility of the ensuing infected cells [76]. It is thus possible that PDGFR cooperates with cAbl during the internalization process but not in subsequent steps.

Independently, Tammer et al. [77] published their evidence suggesting a role for cAbl in H. pylori infection without, however, looking at PDGFR. First, they showed that STI571 and SKI-DV2-43 (an inhibitor often used when resistance to STI571 is encountered) both significantly inhibited tyrosine phosphorylation of CagA compared to untreated cells, despite equivalent CagA protein levels. Consistent with this result, shRNA-mediated depletion of cAbl produced a remarkable decrease in CagA phosphorylation. Interstingly, a simlar depletion of Arg using siRNA yielded an even more impressive reduction of CagA phosphorylation, compared to mock controls, whereas the depletion of both proteins did not reveal an additive effect. In both types of experiments, interfering with cAbl activity or expression resulted in increased cell scattering/motility, which is in agreement with the observations reported by Poppe et al. [76] (see above). Tammer et al. also performed a number of experiments to establish the sequential role of Src and cAbl, as well as the kinetics of phosphorylation of CagA by both enzymes [77]. Perhaps even more interesting though is

their demonstration of the formation of a molecular complex that comprises CagA, CrkII and cAbl, following immunoprecipitation with anti-Abl or anti-CrkII antibodies [77]. This set of experiments clearly shows that the association occurs only in the presence of tyrosine phosphorylated CrkII, which is particularly important as it contradicts the current dogma suggesting that tyrosine phosphorylated CrkII is in a closed inactive conformation. Instead, these results support our findings that tyrosine phosphorylated CrkII does play a positive regulatory role downstream of cAbl [30].

Also related to the investigation of the role of cAbl and CagA in *H. pylori* infection of gastric epithelial cells is the recent paper by Bauer et al. [78]. This study, however, does not focus on internalization of the bacteria themselves through macropinocytosis but rather on what happens to ligand-induced EGFR endocytosis subsequently to host cell infection. Their double immunofluorescence staining showed a dramatic difference in the level of EGFR on the surface of *H. pylori*-infected AGS cells (derived from a human gastric adenocarcinoma), as compared to non-infected controls. This effect was specific to EGFR as the levels of c-Met (an EGFR-related receptor) and HLA-E (an unrelated MHC class I molecule) did not change upon infection. A similar experiment performed on permeabilized cells indicated that the increased level of EGFR on the cell surface was due to inhibition of endocytosis. EGF-EGFR complexes were indeed found to co-localize with EEA1 (a general marker of early endosomes through which proteins transit after endocytosis via the clathrin-dependent pathway) in control non-infected cells [78]. In contrast, there was no detectable co-localization of EGF-bound EGFR molecules and EEA1 in cells that had been infected with *H. pylori*. Importantly, these results were confirmed by flow cytometry. Moreover, once again, HGF-induced endocytosis of c-Met proceeded at the same rate in both infected and control cells, indicating that the inhibitory effect due to *H. pylori* infection is specific to EGFR [78].

In a second set of experiments, the authors investigated the role of the bacterial protein CagA (see above) in the mechanisms leading to inhibition of EGF-induced endocytosis of EGFR. They showed that 1h post-infection, which corresponds to the peak activity of c-Src (responsible for the first/transient tyrosine phosphorylation event on CagA) [77], the presence or absence of CagA had no effect on the surface levels of EGFR. However, 24h after infection (when c-Src is no longer active and cAbl maintains CagA in a permanent phosphorylated state [76, 77]), the absence of CagA prevents the negative effect of *H. pylori* on EGFR endocytosis and, accordingly, restores the surface level back to that found in normal non-infected cells [78]. To further explore the mechanisms underlying the CagA-dependent *H. pylor*i-induced inhibition of EGFR endocytosis, the authors used STI571 and cAbl-specific siRNA to demonstrate that ablation of cAbl activity or expression prevented *H. pylori* from exerting its negative effect on EGFR endocytosis. The rate of EGF-induced EGFR endocytosis in control (non-infected) cells was not affected by either treatment [78]. Taken together, these results thus suggest that cAbl-mediated phosphorylation of CagA is key in the sequence of events that allows H. pylori to block internalization/turnover of EGFR and, presumably, use it to its advantage.

This report [78] is particularly interesting because, not only does it help refine our understanding of how bacteria divert cellular machinery so they can use it to their own benefit, it also supports the evidence discussed earlier that cAbl can negatively regulate endocytosis [20]. It is worth pointing out that the evidence described by Bauer et al. [78] was obtained in the context of endogenous protein expression, i.e. without over-expression of exogenous proteins such as the constitutively active cAbl used by Tanos and Pendergast [20].

The cell system and experimental conditions used by the two groups are thus not exactly the same. However, *H. pylori* infection of the AGS cells used by Bauer et al. [79] did cause a remarkable accumulation of EGFR at the cell surface, which would mimic over-expression of EGFR, as is the case for the NR6 cells [ATCC web site]. Importantly, this non-physiological accumulation of EGFR could very well contribute to the development of gastric cancer from the chronic infection. In any case, the combined results from the literature support the idea that cAbl can act as a positive or negative regulator of endocytosis, depending on the conditions and/or cell types at play.

TYING THE LOOP

It is fascinating to note that the only studies that depict cAbl as a negative regulator of endocytosis so far focused on the ligand-induced internalization of EGFR [20, 78], or an EGF-like receptor [75]. Is it possible that the negative effect of cAbl on internalization is limited to growth factor/growth factor-like receptors? It does not appear to be restricted to EGFR per se. Does it only occur in cells that undergo receptor hyper-signaling as a result of a bacterial infection or mutations in key molecules along the pathway? It will be critical to address these questions by comparing ligand-induced endocytosis of EGFR, PDGFR, and/or other growth factor receptors to determine whether cAbl modulates them similarly. It will be equally important to use the same cell systems in which cAbl was shown to inhibit endocytosis, namely COS7 [20], NR6 [20], or AGS [78] cells, to compare internalization of EGFR and receptors that do not have intrinsic tyrosine kinase activity and thus rely on cytosolic tyrosine kinases (also known as non-receptor tyrosine kinases, or NRTK) to initiate signaling. The fact that CED-1 was negatively regulated by ABL-1 suggests that the effect does not depend on the presence of an intrinsic tyrosine kinase domain. It will also be informative to look at the level of CED-1 in ced-1, ced-2 and ced-10 mutants to see if there is accumulation of CED-1 on the surface of these mutant cells that could lead to hyper-signaling. We should also keep in mind that the evolutionary gap between *C. elegans* and mammalians might explain the difference in this case. Finally, internalization of EGFR should be assessed in non-infected cells that have endogenous levels of both cAbl and EGFR to uncover the effect of cAbl in such conditions. Would cAbl then play a positive role, similar to what we observed for BCR endocytosis in the presence of endogenous levels of cAbl and BCR [30]? There is no doubt that this kind of experiments will be very enlightening.

CONCLUSION

To date, the evidence clearly indicates that cAbl plays a key role in the uptake of viruses and bacteria (both Gran-negative and Gram-indeterminate). To the best of our knowledge, the role of cAbl in internalization of Gram-positive bacteria has yet to be investigated. Does it mean that viral or certain bacterial infections could be treated with cAbl inhibitors such as STI571? This is certainly an appealing possibility that might be especially useful in cases of chronic infection with *H. pylori*, for example, which can progress to cancer. In contrast, it seems that cAbl can act either as a positive or negative regulator of ligand-induced receptor

endocytosis. Whether the outcome depends on the signaling conditions (transformed vs normal cells), the cell type, the receptor involved (endowed with tyrosine kinase activity or not), or the nature of the cargo bound to the receptor, remains to be determined. Either way, the implications for tissue and organ homeostasis are significant. Endocytosis is recognized as a key regulator of cell cycle and cell fate determination, as well as a crucial mechanism of signaling attenuation, which itself is critical to prevent hyper-signaling and cell transformation [reviewed in 80]. Endocytosis allows for both temporal and spatial restriction of receptor signaling. Deregulation of receptor signaling (e.g. EGFR, Notch) is of established relevance in cancer. Likewise, deregulation of cAbl (through Bcr-cAbl fusion events) is well known to drive neoplastic transformation leading to leukemia. Recent observations indicate that cAbl is also deregulated in solid tumors though. Specifically, high kinase activities and/or protein levels have been reported in cases of thyroid cancers, breast carcinomas, and non-small cell lung cancers [reviewed in 1]. Interestingly, cAbl fusion proteins have not been detected in solid tumors isolated from patients. Instead, two separate high throughput analyses of various tumors and cancer cell lines revealed the presence of deletions and/or somatic mutations in cAbl mRNA [1]. Most of these mutations/deletions appeared to be localized in the kinase domain, the Cap region (a small conserved region at the N-terminal end of the protein which interacts with the SH2 and SH3 domains to clamp the kinase domain in an inactive confirmation), and the SH2 domain. The fact that the SH2 domain was found to be mutated *in vivo* is of particular interest because it validates the *in vitro* results showing that over-expression of constitutively active cAbl (obtained by mutating Pro residues in the SH2 domain) inhibits ligand-induced EGFR endocytosis [20]. Could wt and mutated cAbl really have opposite functions then? Does mutated cAbl modulate endocytosis in tumors *in vivo*? Is the deregulation of cAbl found in tumors exclusively associated with the tumor cells per se? Is it present in stromal cells as well? These are important questions that require more experimentation. In particular, the latter question could turn out to be highly informative given the recent paper by Wilkes and Leof [80] who showed that activation of a subpopulation of cultured fibroblasts depends on cAbl activity. Considering the emerging importance of activated fibroblasts (also known as stromal fibroblasts, or reactive fibroblasts) in cancer [reviewed in 81], it will be fascinating to determine how cAbl functions in those cells. Does it regulate endocytosis at all? If so, does it promote or inhibit it? Does it play the same role in tumor and stromal cells? Similarly, a role for cAbl in inducing the transition from fibroblasts to myofibroblasts would be expected to have a major impact on fibrotic diseases such as interstitial pulmonary fibrosis (IPF), liver cirrhosis, etc. There is no doubt that the answers to these questions will help refine our understanding of the mechanisms of endocytosis in general, and since internalization is central to so many cellular processes and, by extension, to so many types of cancer and infections, it will likely help us design better therapies to target infections and cancer as well.

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Chapter 5

CELLULAR UPTAKE MECHANISM OF INORGANIC NANOVEHICLES FOR DRUG DELIVERY: STRUCTURAL FEATURES AND ENDOCYTIC PATHWAY

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ABSTRACT

In this review, the cellular uptake mechanism of inorganic nanoparticles, as drug delivery carrier, is discussed, particularly focusing on layered double hydroxide (LDH). The LDH nanoparticles are biocompatible in general consisting of positively charged metal hydroxide sheets and charge-compensating interlayer anions, which are solvated with water or solvent molecules depending upon synthetic conditions. Such inorganic nanoparticles have attracted a great deal of attention nowadays as nanovehicles due to their efficient delivery behaviors for drugs or genes into cells. Specific internalization pathway, cellular uptake rate, and delivery efficiency of LDHs will be described in details with respect to their structural features such as particle size (50, 100, 200 and 350 nm) and surface modification (folate conjugates). Cellular entry of other inorganic nanoparticles including carbon nanotube, iron oxide and silica will be also discussed with LDHs comparatively. And finally attempts will be made to understand the interaction mechanism between delivery nanovehicles and cells, because it will provide a new

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perspective for the design of drug delivery nanovehicles with maximized and targeted delivery ability.

INTRODUCTION

During past decades, a wide variety of nanomaterials such as nanoparticles, nanoemulsions, nanocomposites or nanohybrids, and nanoporous ones have been extensively applied to industrial fields and human-related products.[1-10] In particular, the use of nanomaterials in medicinal fields, namely nanomedicine, as molecular diagnostic agents or drug delivery systems (DDS) has attracted a great attentions.[1, 4, 11-18] In the beginning of nanotechnology researches and developments, nanostructured materials in the physico-chemical point of view have been mainly developed. In other words, the structure-property relationships of various inorganic, organic and biomolecules were determined in nanometer scale, aiming at exploring novel properties and functions. Diverse types of nanostructures (aerosols and colloids) and products physically or chemically modified with nanomaterials (coatings, nanocomposites, and nanohybrids) have been synthesized as 1st generation nanomaterials.[19-21] However, the practical application of such nanostructured materials as DDS was often limited due to their low delivery efficiency to the target organs like tumor tissue. Recently many researches have focused on the design of functional nanostructures, so-called 2nd generation nanomaterials, by modulating structure and intentionally conjugating functional groups which facilitate ligand-receptor interaction on the cell surface of the target tissue.[19, 22-25] For example, since antibody specifically recognizes tumor antigen or ligands which interact with tumor cell overexpressing receptors, it can be conjugated to nanostructured delivery carriers to induce target specific receptor-mediated endocytosis.[26-31]

Nowadays, various inorganic nanoparticles for drug delivery carriers have been extensively studied in terms of delivery efficiency, drug loading capacity, controlled drug release and in vivo systemic circulation. Nevertheless, the factors influencing cellular uptake, internalization pathway, cellular fate of nanoparticles and subsequent signal transductions are remained unelucidated. The interaction between nanomaterials and biological systems at cellular and molecular levels as well as their intracellular trafficking pathways must be understoodis essentially needed, which can lead to develop new strategies for the design of nanomedicine with high delivery efficiency and drug efficacy and etc.

In this chapter, we are going to describe cellular uptake mechanism and delivery efficiency of inorganic layered materials for drug or gene delivery and their relationship with followed by the structural features influencing on cellular uptake pathway.

1. LAYERED METAL HYDROXIDE MATERIALS

Anionic clays, layered double hydroxides (LDHs), have a wide range of chemical compositions and their layered structures exhibit a variety of stacking faults to generate different polytypes. LDHs have hydrotalcite-like structure and their general formula can be expressed as $[M^{2+}_{1-x}M^{3+}_{x}(OH)_2]^{+}(A^{m-})_{x/m},nH_2O$, where the M^{n+} are metal cations ($M^{2+} = Mg^{2+}$, Zn^{2+}, Ni^{2+}, Cu^{2+},…, and $M^{3+} = Al^{3+}$, Fe^{3+},…) and A^{m-} stands for interlayer anions (A^{m-} =

CO_3^{2-}, NO_3^-, SO_4^{2-}, and other anionic species).[32-37] The isomorphous substitution of M^{2+} ions with M^{3+} ones results in the positive charge on brucite-like layer and thus LDHs can accommodate charge-compensating anions in the interlayer space (Figure 1). Compared to the cationic clay, LDHs have high layer charge densities (2-5 meq/g), giving rise to a strong electrostatic interaction between the brucite-type sheets and the anions. So one can expect that multivalent anions can energetically be better stabilized within their interlayer space than the monovalent due to the gain of higher lattice energy. It is, therefore, not that surprising that LDHs with monovalent anions like nitrate or chloride ions can be good precursors for exchange reactions because of their weaker ionic interaction between the brucite sheets.

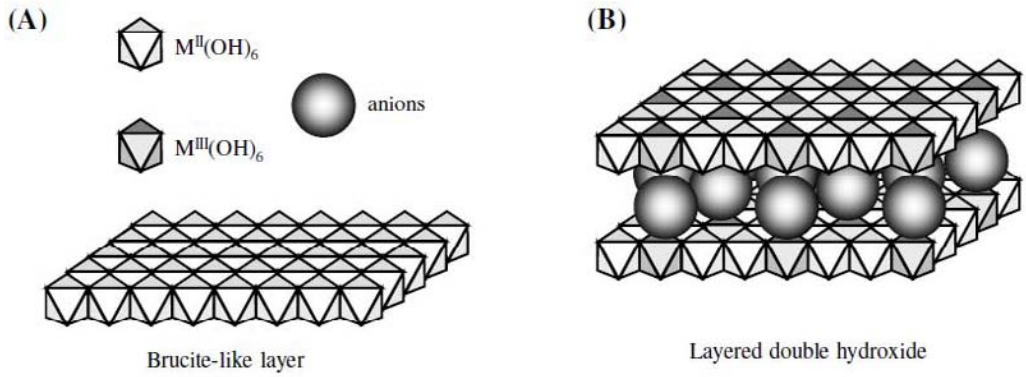

Figure 1. Schematic structure of brucite-like layers (A) and layered double hydroxide (B). Electrostatic interaction between the positive charged layer, $M^{II}_{1-x}M^{III}_x(OH)_2^{x+}$ and interlayer anions stabilizes the lamellar structure of LDHs.

As described above, the interlayered anions in LDHs are in general easily exchangeable. In this regards, LDHs are very attractive to intercalate, stabilize and reorganize anionic labile molecules under physic-chemical or physiological conditions. Bioactive molecules with negative charge such as DNA, nucleotides, vitamins, drugs and food ingredients can be encapsulated into 2-dimensional LDH lattice.[3, 5, 38-44] When these biomolecules are delivered by LDHs in the biological systems, they can be easily exchanged with various anions like chloride and/or carbonate ions abundantly present in the body. In addition, the pH dependent solubility of LDHs is one of the most fascinating properties of LDHs for biological applications. LDHs are soluble in slightly acidic conditions like endosome (pH 5.5 – 6.0) and lysosome (pH 4.5 – 5.0) to release interlayer biomolecules. In other words, LDHs can protect intercalated biomolecules on the cell surface with high efficiency because pH around lipid bilayers, which constitute the plasma membrane, is about 7.0 at which LDH is not solubilized. When LDHs penetrate the cell membrane, they can be then gradually solubilized in the endosome and finally dissolved in the lysosome, thereby releasing intercalated biomolecules into the cellular organelles in a controlled manner. The decomposed LDHs, as a form of metal ions, may be easily secreted from the cells after delivering biomolecules to the target organelle.

2. SYNTHESIS OF DRUG-LDH NANOHYBRIDS

Generally, LDHs are synthesized by coprecipitating the mixed cations in a solution upon base titration. On the other hand, various synthetic approaches can be utilized for the intercalative hybridization between LDHs and drug molecules, including coprecipitation, anion exchange, and reconstruction. In coprecipitation reaction, a mixed solution containing M^{2+}, M^{3+} and anionic drug molecules is titrated with an alkaline solution. The coprecipitation reaction is considered as the most simple and convenient, but can not be applicable if metal ions and anionic drug molecules form comlex clusters before coprecipitation. LDHs with exchangeable interlayer anions, such as NO_3^- and Cl^-, could be subjected to ion exchange reactions. The anionic drug molecules outside of LDH diffuse into the interlayer space through concentration gradient. In some cases, the reconstruction reaction can be used for drug-LDH hybrids. Upon heating to ~ 150 °C, the LDH is dehydrated by losing its surface adsorbed and interlayer water molecules successively. In the temperature range of 400 ~ 500 °C, the dehydroxylation of metal hydroxide layer and the decomposition of interlayer anion occur along with the formation of mixed oxides Thus formed mixed metal oxides, when dispersed in an aqueous solution containing appropriate drug molecules, are rehydrated, and recrystallized into LDHs upon intercalation of drug molecules, which occur simultaneously.[45]

LDH nanoparticles with a formula of $Mg_{0.68}Al_{0.32}(OH)_2(CO_3)_{0.16}{\cdot}0.1H_2O$ are easily produced by conventional co-precipitation route; a mixed solution containing both $Mg(NO_3)_2{\cdot}6H_2O$ (0.032 M) and $Al(NO_3)_3{\cdot}9H_2O$ (0.016 M) is titrated with strong base NaOH solution (0.5 M) containing $NaHCO_3$ (0.5 M) at room temperature.[13, 46-49] The final pH of the solution is then adjusted to 9.5 ± 0.2 at which co-precipitation occurs. The resulting white precipitate is collected by centrifugation and washed with deionized water thoroughly. Diverse types of anionic biomolecules can be effectively intercalated into the flexible gallery space of LDHs via co-precipitation or ion-exchange reaction, which is finally stabilized by electrostatic interaction.

In the same way, drug-LDH nanohybrids can be also synthesized by co-precipitation method.[48, 50, 51] In order to produce an anticancer drug, methotrexate (MTX) encapsulated by LDH (MTX-LDH), either coprecipitation or anion exchange reaction is utilized. For coprecipitation, powdered MTX is dissolved in decarbonated water, and titrated with NaOH solution (0.5 M) to induce anionic moiety at pH 7.0. And a mixed metal solution of $Mg(NO_3)_2{\cdot}6H_2O$ and $Al(NO_3)_3{\cdot}9H_2O$ with a molar ratio of Mg/Al = 2/1 is then added to MTX solution. This solution is titrated with a NaOH solution until pH ~ 9.5 to produce yellowish precipitates. The suspension is stirred at room temperature for 48 h under nitrogen purging, and then the resulting yellowish precipitate is isolated by centrifugation, washed with deionized water, and freeze-dried. For anion exchange reaction, the pristine $Mg_2Al(OH)_6(NO_3)$-LDH is first synthesized by coprecipitation method, and then they are dispersed in an aqueous MTX solution with a MTX/Al molar ratio of 1.5. Thus prepared bio-inorganic hybrids can be confirmed by X-ray diffraction patterns, since the interlayer distance of 2-dimensional LDHs changes upon the size of biomolecules intercalated. As shown in Figure 2, the d-spacings of the pristine LDH and MTX-LDH hybrid are determined to be 8.5 and 20.8 Å, respectively, indicating that intercalation of MTX into LDH lattice giving rise to an increase of basal spacing. The longitudinal molecular length of MTX (~23 Å) is turned out to be larger than the gallery height of MTX-LDH (16 Å), considering the layer thickness of

LDH (4.8 Å). This suggests that MTX molecules are closely packed with a slightly tilting angle of ~44°.

5-fluorouracil-LDH nanohybrid (5-Fu-LDH) is also prepared by either coprecipitation or reconstruction route.[12] For coprecipitation, the powdered 5-Fu is dissolved in a decarbonated water (0.032 M) and 0.5 M NaOH solution is added dropwise until the pH becomes ~8.0. And a mixed solution of $Mg(NO_3)_2 \cdot 6H_2O$ and $Al(NO_3)_3 \cdot 9H_2O$ is then added to 5-Fu solution. The final pH of the mixture is adjusted to 9.5 by adding an aqueous solution of NaOH as describe above. In the reconstruction reaction, $Mg_2Al(OH)_6(CO_3)_{0.5}$-LDH is calcined at 450 ℃ for 4 h. The resulting metal oxide $Mg_2AlO_{3.5}$, is directly dispersed in a 5-Fu solution with a 5-Fu/Al molar ration of 1.5. According to the X-ray diffraction analysis, the basal spacing of thus prepared 5-Fu-LDH is determined to be 10.6 Å, indicating that the pristine LDH lattice (NO_3-LDH:4.8 Å) is clearly expanded upon intercalation of 5-FU (Figure 2). This result is quite consistent with the sum of the thickness of LDH (4.8 Å) and the longitudinal van der Waals radii of 5-Fu (5.8 Å), suggesting successful intercalation.

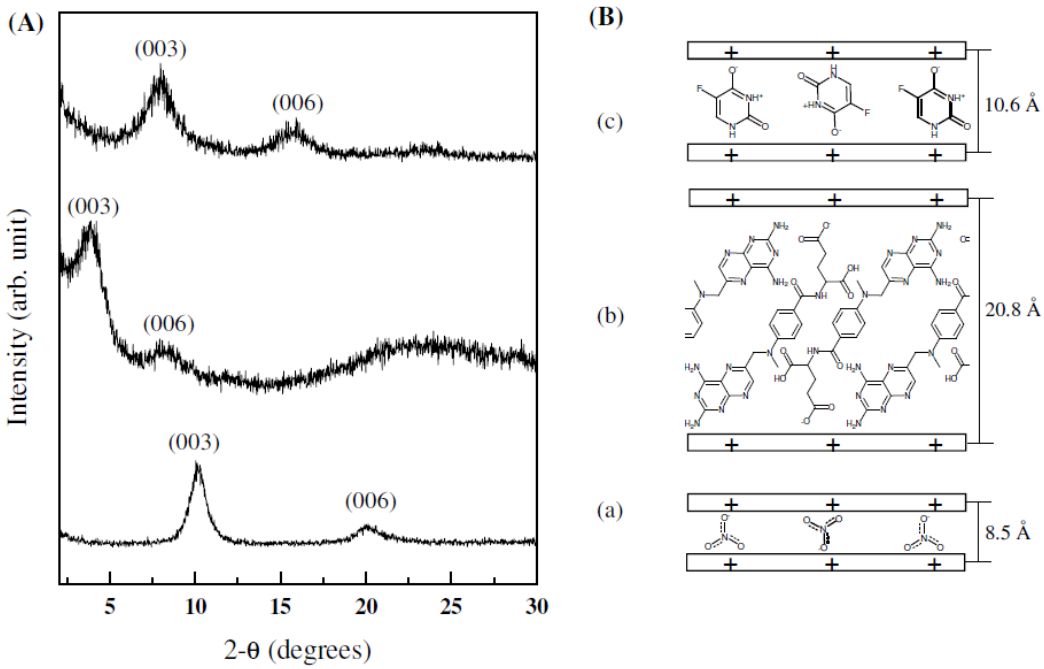

Figure 2. X-ray diffraction patterns (A) and schematic illustration (B) for intercalates pristine LDH (a), MTX-LDH (b) and 5-Fu-LDH (c).

The particle size of LDH can be precisely controlled on the basis of crystal growth theory. The crystal growth of LDH nanoparticles occurs in two steps. First the nuclei of seed nanoparticles are developed (nucleation step) and then the seed particles grow upon further aging in nutrient solution (crystal growth step). Generally the particle size at the equilibrium is dependent on the concentration and the number of seed; $L \propto (c/N)^{1/3}$ where L is particle size, c nutrient concentration, and N the number of seed nuclei).[52] And the crystal growth rate can be expressed as $dL/dt = C \cdot \exp(-1/RT)$ (particle size L, time t, nutrient concentration C, temperature T, gas constant R). Therefore, the particle size of LDHs can be controlled by

varying the reaction conditions such as nutrient concentration, reaction temperature and time. Based on above theory, the LDH particles are prepared with average sizes of 50, 100, 200 and 350 nm, namely by controlling the reaction time and temperature as follows; LDH of 50 nm in size can be prepared by coprecipitating Mg^{2+} and Al^{3+} ions in an aqueous solution upon NaOH titration at room temperature, and by aging for 12 h successively. For LDHs with 100, 200, and 350 nm in sizes, the reaction temperature and time are controlled to maintain at 100°C for 12 h, 200°C for 24 h, and 200°C for 48 h, respectively. As shown in Figure 3, the longer the reaction time and the higher the temperature are, the bigger the particle size becomes. One thing to be noted here is that the LDH nanoparticles thus prepared show narrow size distribution.

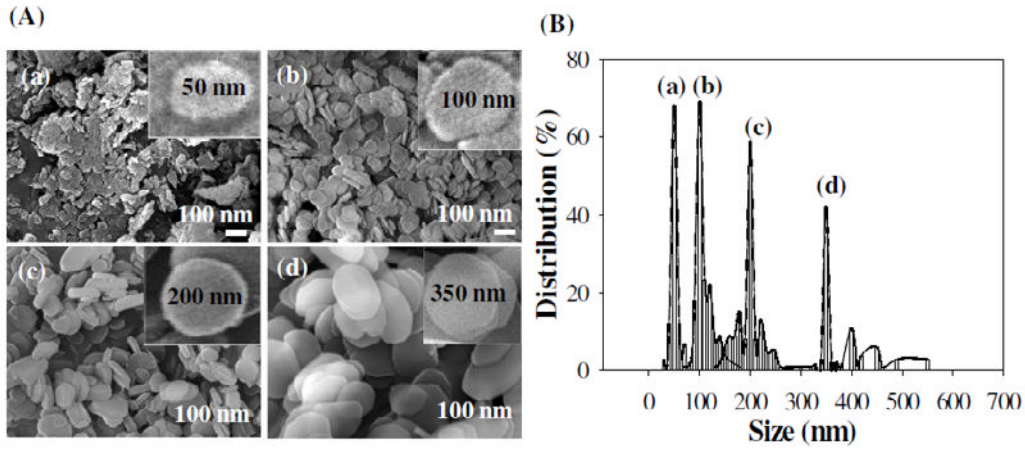

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Figure 3. Field emission scanning electron microscope (FE-SEM) images (A) and the corresponding histogram of particle size distribution (B) for LDHs with 50 nm (a), 100 nm (b), 200 nm (c) and 350 nm (d). For the histogram, 200 grains were randomly selected from SEM images of each sample and the sizes were measured particle by particle. The inset images in (A) are the magnification of one particle.

3. Cellular Uptake Mechanism of Layered Double Hydroxides

Essential nutrients, small molecules, such as amino acids, sugars and ions can actively traverse the plasma membrane through integral membrane pumps or channels. Macromolecules, however, could be transported into cells through a process called endocytosis, a conserved process in eukaryotes by which extracellular components are taken up into cells. During this process, an invagination of the plasma membrane occurs to form vesicles that enclose the extracellular materials. In general, endocytosis can be broadly divided into two categories based on the materials internalized; phagocytosis and pinocytosis. Phagocytosis or “cell eating”, the uptake of large solid particles (0.25 – 10 μm) such as bacteria, is performed by specialized cells such as macrophages, monocytes and neutrophils

to remove pathogens and cell debris, and plays an important role in the immune system. Pinocytosis or "cell drinking", a uptake of fluids including all solutes present, occurs in all mammalian cell types and this process requires energy in the form of adenosine triphosphate (ATP). In contrast to phagocytosis which forms an internal phagosome or "food vacuole", pinocytosis generates very small vesicles which subsequently fuse with lysosomes to hydrolyze the particles taken. Pinocytosis is mediated by at least four basic mechanisms: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis.[53, 54] The macropinocytosis is induced in many cell types to form a pocket in a nonspecific manner where the large ruffles on the plasma membrane, generally larger than 1 μm in size, are formed. The clathrin-mediated endocytosis occurs in all mammalian cells and is the most common uptake mechanism for essential nutrients such as low-density lipoprotein and transferrin. Receptor binding triggers clathrin protein recruitment and/or assembly on the cytoplasmic surface of the plasma membrane as it begins to invaginate, forming clathrin-coated vesicles with a size of about 120 nm.[53, 55] The coat is composed of clathrin heavy and light chains that assemble into three-legged triskelions.[56,57] The caveolae-mediated endocytosis is processed by caveolae, flask-shaped small invaginations (~ 60 nm) of the plasma membrane in many vertebrate cell types, especially in endothelial cells where cholesterol and sphingolipids are extremely abundant.[58, 59] The shape and structural organization of caveolae are referred as caveolin, a diametric protein that associated with cholesterol and sphingolipids.[60] The last pinocytic pathway, the clathrin- and caveolae-independent endocytosis is described only in negative terms. The mechanisms that govern clathrin- and caveolae-independent endocytosis remain to be unclearly elucidated, but one of the most well known process involves lipid 'rafts'-mediated endocytosis.[60] The difference between caveolae and 'rafts' is that caveolae represent just one type of cholesterol-rich microdomain on the plasma membrane, while lipid 'rafts' have small structures, 40-50 nm in diameter, that do not contain caveolin, but can be isolated from all types of cells.

In the view point of biology, the essential requirement of nanoparticles for pharmaceutical and medical applications such as MRI, hyperthermia treatment, gene therapy and chemotherapy, is that nanoparticles should efficiently traverse the cell membrane, which is most likely via endocytosis or pinocytosis. As previously studied in detail, the specific endocytic pathway for LDH nanoparticles has been clearly demonstrated by showing the cellular membrane entry with immunofluorescence and confocal microscopic analyses.[46] These two methods are, in general, known to be very useful for tracing intracellular trafficking and identifying components on the endocytic pathways. For the immunofluorescence microscopic study, tumorogenic osteosarcoma MNNG/HOS cells are treated with green fluorescent fluorescein 5'-isothiocyanate (FITC) loaded LDH (FITC-LDH) for 2 h, since the highest cellular uptake of FITC-LDH with the maximum fluorescence intensity can be observed after incubation for 2 h. The cells are then stained with either anti-clathrin antibody or anti-caveolin-1 antibody both conjugated to red fluorescent Texas Red (TR) and subsequently observed by fluorescence microscopy. As shown in Figure 4, the clathrin proteins stained by red fluorescence are well expressed and perfectly overlapped with green fluorescent FITC-LDH. On the other hand, the caveolin-1 is observed very faintly, and does not colocalized with FITC-LDH (Figure 4A). According to the confocal microscopic images, FITC-LDHs are mainly found in the cytoplasm with perinuclear localization (Figure 4B). The magnified confocal images clearly demonstrate the overlapped localization of FITC-

LDH with clathrin, which is well observed by a yellow merged fluorescence (Figure 4C). This result suggests that the clathrin-mediated endocytosis is principally responsible for the internalization of LDH nanoparticles into cells, while the role of caveolae is not important for LDH uptake.

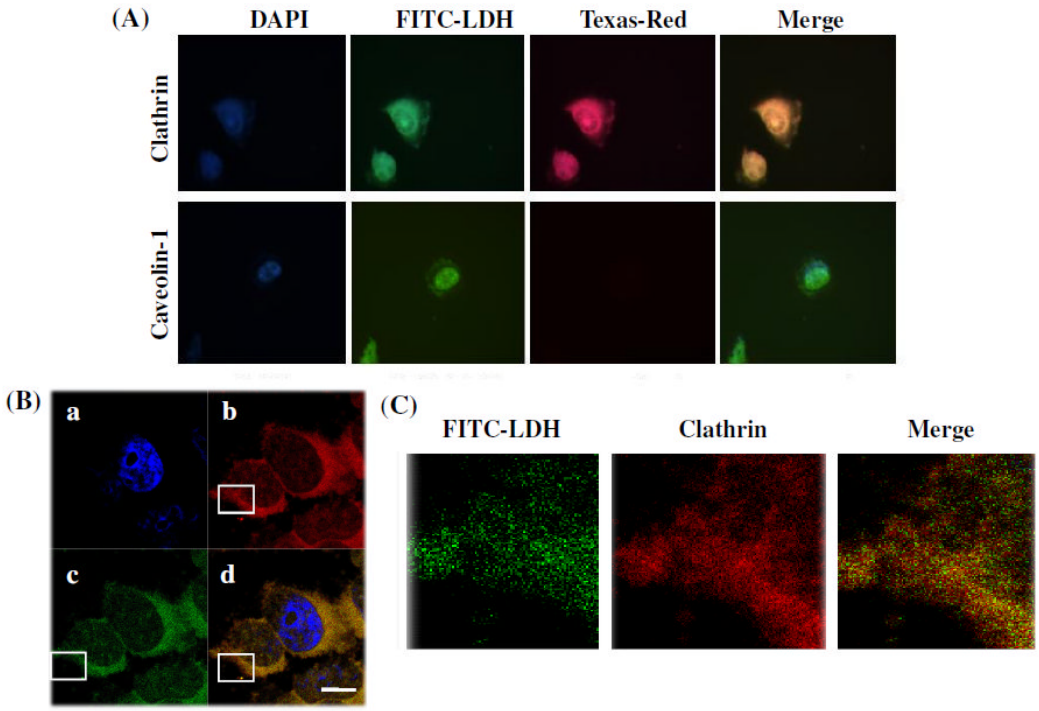

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Figure 4. (A) Immunofluorescence microscopic images showing the colocalization of clathrin and LDH in MNNG/HOS cells. (B) Confocal microscopic images of FITC-LDH and clathrin in MNNG/HOS cells. Localization of (a) nucleus, (b) clathrin and (c) FITC-LDH, the merged image (d) in MNNG/HOS cells. Cells were incubated with FITC-LDH for 2 h, treated with clathrin antibodies and stained by TR and DAPI. Scale bar represents 10 μm. (C) Magnified images of the white boxes in (B) are also given. The images identify areas showing colocalization of FITC-LDH and clathrin, as shown merged in yellow.

The clathrin-mediated endocytosis of LDH nanoparticles is also verified by cross-checking the involvement of some accessory proteins, playing a role for assembling clathrin-coated pits such as dynamin and eps 15.[46] Dynamin is a GTPase enzyme required for the fission of endocytic vesicles from the plasma membrane.[61], which regulates membrane trafficking events at the cell surface, and is required for phagocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis and some clathrin- and caveolae-independent endocytic pathways.[62] Eps 15 binds the plasma membrane adaptor protein, AP2, and is implicated in regulating clathrin-coated pit assembly.[63] Microscopic images of MNNG/HOS cells incubated with FITC-LDH, followed by staining with antibodies against clathrin, dynamin and eps 15, all conjugated to red fluorescent Alexa Fluor 549, show that FITC-LDHs are well colocalized not only with clathrin, but also with dynamin and eps 15 (Figure 5A). In particular, the same localization of FITC-LDH with eps 15, which is only involved in

clathrin-mediated endocytosis, strongly supports the role of clathrin for LDH uptake. When transferrin labeled with red Alexa Fluor 594 is used as a positive control and its localization is compared with FITC-LDH, high colocalization between FITC-LDH and transferrin could be also observed (Figure 5B), indicating the same cellular entry of LDH to that of transferrin.

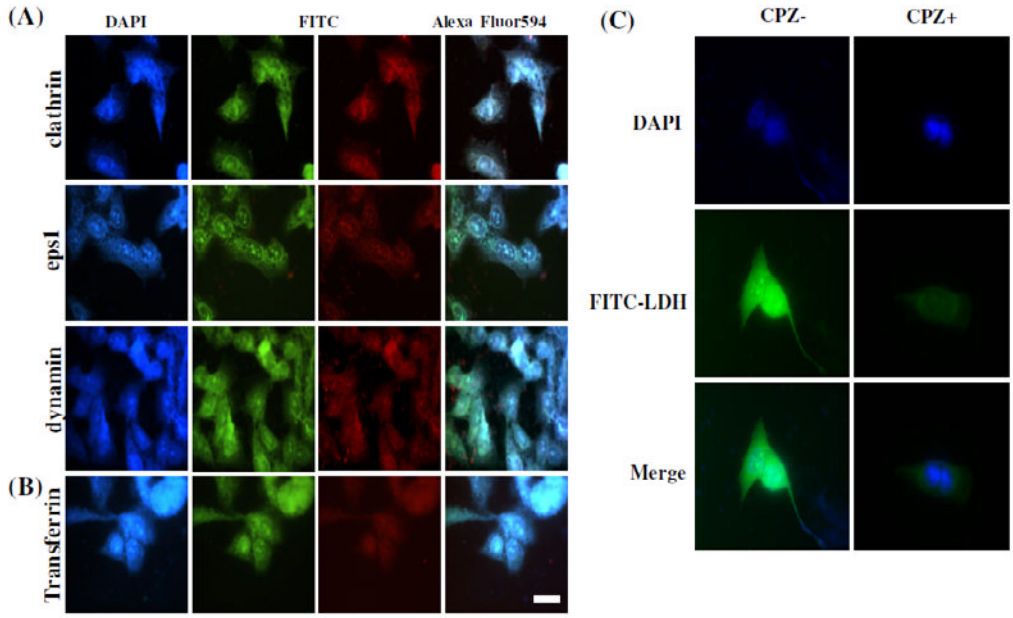

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Figure 5. Colocalization of FITC-LDH with clathrin-mediated endocytic proteins and markers. FITC-LDH-treated MNNG/HOS cells were fixed and processed for immunofluorescence microscopy. (A) Fluorescence microscopic images of the cells treated with clathrin, eps15 and dynamin antibodies and then stained with secondary antibodies conjugated to Alexa Fluor 594, showing the same localization pattern. (B) Colocalization of FITC-LDH and Alexa Fluor 594-labeled Tf, a well-known marker of clathrin-mediated endocytosis. The localization of LDH and Tf clearly overlaps. Scale bars, 20 μm. (C) The effect of clathrin inhibition on the entry of LDHs in MNNG/HOS cells. The cells initially incubated with a clathrin inhibitor, CPZ, showed a considerable reduction in LDH particle uptake.

In addition, cellular uptake mechanism can be determined by employing specific endocytosis inhibitors, followed by examining the modulation of uptake amount. The LDH entry via clathrin-coated vesicles is biochemically confirmed by pre-incubating the cells with chlorpromazine (CPZ), a clathrin inhibitor that disrupts the assembly of the clathrin adaptor protein at the cell surface.[64] And, then the uptake change of FITC-LDH is observed by measuring fluorescence intensity. As expected, the inhibition of clathrin-coated pits leads to a dramatic decrease in the LDH uptake (Figure 5C), which is in good agreement with previous conclusion on clathrin-mediated internalization of LDH nanoparticles. Intracellular trafficking mechanism of LDH can be also predicted, since particles taken up by clathrin-coated vesicles are known to be transported to endosomes and subsequently to the Golgi complex or/and lysosomes after uptake.

Recent studies have demonstrated that certain inorganic nanoparticles enter cells via energy-dependant endocytic pathway, whereas, most of organic nanoparticles use fusion-like mechanism and do not use energy for their internalization into cells.[65] The general cellular

uptake mechanism for inorganic nanoparticles through endocytic pathway is now well documented and accepted. The silica-coated nanoparticles are determined to be transported into HeLa cells in part through adsorptive endocytosis and in part through clathrin-coated pit formation.[66] The carbon nanotubes as intracellular protein transporters are internalized into the cytoplasm of cells via endocytosis pathway, releasing the proteins in the endosome.[67,68] The cellular uptake of silica-overcoated magnetic nanoparticles is reported to be mediated through energy-dependent endocytosis in A549 cells.[69]

The fact that the LDH nanoparticles enter cells via clathrin-mediated endocytic process is very attractive for biological and pharmaceutical purpose, since the clathrin-mediated endocytosis occurs constitutively in all types of mammalian cells and plays a fundamental role in many cellular activities. Therefore, the cellular uptake of LDH can be actively facilitated, which is one of the most important critical properties as delivery carriers.

4. Relationships Among Uptake Mechanism, Delivery Efficiency and Drug Efficacy

The clathrin-mediated endocytosis of LDH nanoparticles is surely be correlated with their delivery efficiency, because LDHs are easily taken up by cells, thereby drug intercalated and encapsulated in LDH can be effectively transported into cells. When an anticancer drug, MTX, is encapsulated in LDH, the MTX-LDH nanohybrid can be prepared, as described above in section 2. Since MTX acts as a folate antagonist and binds competitively and reversibly to dihydrofolate reductase, the folate cycle in the cell could be blocked. And as a consequence, the synthesis of thymidine, purine, DNA, RNA and proteins could be inhibited.[70] However, the clinical use of MTX is often limited in various cancer treatments because of its toxic side effects related to high dose MTX therapy. The fact that MTX possesses antifolate property in a competitive manner with folate is one of the major disadvantages of MTX, requiring its relatively high dose for practical treatment.[71] And, negatively charged property of MTX in physiological condition induces repulsive force between MTX and the negatively charged plasma membrane, resulting in low cellular uptake. Therefore, we believe that MTX-LDH nanohybrid system can overcome such obstacles by enhancing nanoparticles-cellular interaction.

In order ot demonstrate such interactions, the MTX-LDH is treated on MNNG/HOS cells and the intracellular level of MTX is quantified by using HPLC to determine drug delivery efficiency of LDH carriers.[48] Cells (1×10^6) are seeded on a 60 mm culture dish and treated with 50 μM/ml MTX or 112 μg/mL of MTX-LDH, where the concentration of MTX in MTX-LDH is adjusted to maintain equivalent molar amount of the free MTX-treated. The concentration of MTX in the cells delivered through MTX-LDH is remarkably higher than in the cells incubated with free MTX only (Figure 6A). It is worth to note here that the MTX concentration in free MTX only-treated cells is determined to be very low and barely detectable by HPLC analysis. The enhanced cellular uptake of MTX using MTX-LDH hybrid is also confirmed by flow cytometry with FITC conjugated MTX (MTX-FITC) or MTX-FITC intercalated into LDH layer (MTX-FITC-LDH). The cellular uptake of MTX is about 55-fold higher for MTX-FITC-LDH than for free MTX-FITC after incubation for 1 h and 2 h as determined by fluorescence intensity (Figure 6B). These results clearly indicate that the

MTX-LDH nanohybrid system can enhance the internalization of encapsulated drug molecules, which is strongly associated with the efficient clathrin-mediated endocytic pathway of LDH carrier. The large difference in intracellular MTX level between free MTX and MTX-LDH can also be an evidence that the LDH nanoparticle could play a role as an excellent carrier.

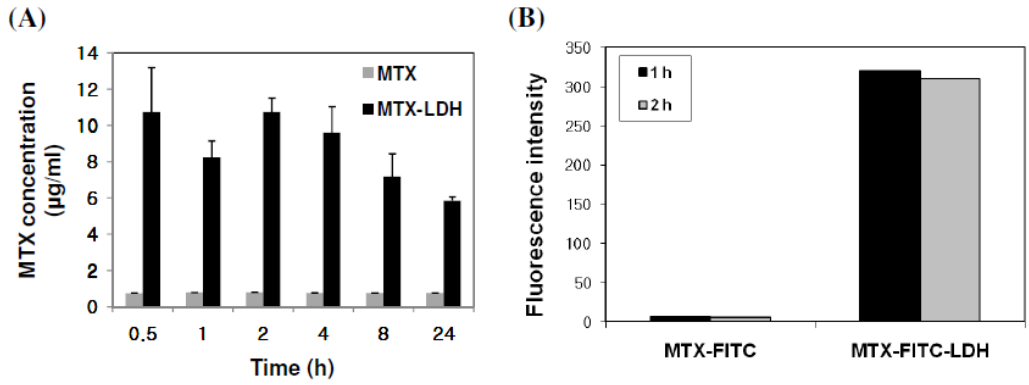

Figure 6. Cellular accumulation of free MTX molecules in MNNG/HOS cells treated with either MTX or MTX-LDH as quantified by HPLC analysis (A) and flow cytometry (B).

The increased drug accumulation in the cells may enhance drug efficacy as well. Effects of MTX-LDH on cancer cell proliferation are evaluated in MNNG/HOS cells by MTT assay.[48] It is based on the reduction of the yellow tetrazolium salt MTT by metabolically active cells, resulting in purple formazan crystals. It is, therefore, not that surprising that MTX-LDH can significantly inhibit the cancer cell proliferation, with half maximal inhibitory concentration, IC_{50} values of 10.21 ng/ml for MTX-LDH versus 3675.12 ng/ml for free MTX only (Figure 7A). This result suggests that MTX-LDH effectively acts as a cancer chemotherapy agent, inducing enhanced efficacy compared to free MTX, which is strongly related to the specific membrane penetrating capacity of LDH carrier via clathrin-mediated endocytosis. Anticancer drug efficacy of MTX-LDH can also be evaluated for other cancer cell lines such as human lung adenocarcinoma cells (A549) and liver carcinoma cells (Hep1), and be determined to be strongly enhanced as compared to free MTX, although IC_{50} values are dependent on the cell lines tested.[12]

The delivery efficiency and drug efficacy are also assessed by employing another drug-LDH nanohybrid, 5-Fu loaded LDH (5-Fu-LDH).[12] 5-Fu is widely used against many cancers and belongs to the family of antimetabolite drugs. Its mode of action is related to the suppression of cancer cell growth by deactivating the pyrimidine synthase, and this leads to block synthesis of the pyrimidine, a nucleotide required for DNA replication.[72] IC_{50} values for 5-Fu-LDH are determined to be about 2.5 to 4.3 fold lower than that for free 5-Fu depending on the cancer cell lines tested (Figure 7B). All the results we have made clearly demonstrate the efficient delivery function and enhanced efficacy of anticancer drug-LDH nanohybrids in cultured cell lines.

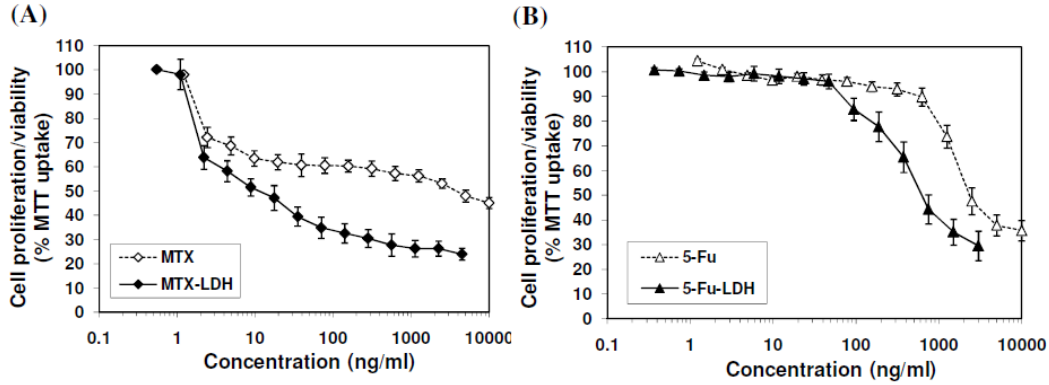

Figure 7. Effects of free drugs or drug-LDH nanohybrids on the inhibition of MNNG/HOS cell proliferation after 72 h.

The targeted cell entry of drug delivery nanoparticles via specific membrane interactions is one of the most critical factors for their practical biological applications. The administered anticancer drug should easily access to principally tumor tissue, not normal tissue, in order to minimize size effects and increase therapeutic effects as well. In this context, some nanoparticles conjugated to transferrin are developed to induce transferrin ligand/receptor interaction on the cell surface, since transferrin is specifically recognized and internalized by transferrin receptors-mediated endocytosis. It has been also reported that transferrin receptors are massively expressed on the surface of many types of tumor tissues.[73] The transferrin-receptor interaction has, therefore, attracted much attention as a potential uptake pathway for the efficient delivery of drug or gene molecules into tumor cells.[74-76] It should be noted that transferrin receptor mediates the endocytosis of transferrin through clathrin-dependent process. Indeed, our results demonstrating that LDH nanoparticles mainly enter cells via clathrin-mediated internalization mechanism such as the transferrin-receptor interaction suggest that they may be exploited as promising drug delivery nanovehicles.

5. Factors Influencing on Uptake Mechanism

5.1. Structural Feature: Particle Size

Many physico-chemical properties of delivery nanocarriers affect cellular interaction, including particle size, shape, surface charge and etc. Among them, the particle size is considered as a critical factor for the interaction with the plasma membrane, uptake mechanism and subsequent intracellular processing. In the viewpoint of endocytic invagination, the sizes of macropinocytic membrane ruffling, clathrin-coated vesicles and caveolae are about > 1 μm, 120 nm and 60 nm, respectively, which may also influence on the selection of the particles uptaken. To be effective in the systemic circulation, the optimum size of drug delivery nanovehicles is considered to be less than 200 nm in order to bypass non-selective uptake by macrophages of reticuloendothelial system actively developed in the

liver and spleen. But, particles smaller than 5 nm are likely to be easily eliminated by rapid clearance in the kidney.

Some researches have investigated the particle size effects on cellular uptake and endocytosis pathway.[77] A quantum dot conjugated sugar ball shows highly size-dependent endocytosis (50 > 15 > 5 nm) and its optimal size is suggested to be around 50 nm.[78] According to the study on the size effects of biodegradable polymeric particles on cellular uptake, the particles with 100 nm in size induce higher cellular uptake when compared to larger particles such as 300, 600, 1000 and 2000 nm.[79] The size-dependent cellular uptake of single-walled carbon nanotube (SWCNT) and the Au nanoparticles have been also reported, proving maximal uptake at a common radius of 25 nm in NIH-3T3 cells.[80] However, most of these studies have focused on the simple determination of optimum size of particles for efficient cell penetration. Therefore, little information is currently available about the size effects of inorganic nanoparticles on internalization pathway.

According to our study, attempts have been made more precisely to determine the relationships between LDH particle size and uptake mechanism. LDH nanoparticles with uniformly controlled sizes of 50 nm, 100 nm, 200 nm and 350 nm have been successfully prepared under hydrothermal condition.[49] And the fluorophore, FITC, is conjugated to the surface of each LDH on the basis of crystal growth mechanism and inorganic-organic coupling reaction to effectively trace LDH nanoparticles in the cells. The uptake rate of LDHs by MNNG/HOS cells is determined to be highly size-dependent (50 > 100 – 200 > 350 nm) after 2 h incubation. One thing to be underlined here is that little amount of 350 nm could be internalized into cells (Figure 8). This in an indication that LDH particles in the size range of 50 to 200 nm can easily penetrate the cell membrane, but those of 350 nm is too large to massively enter cells. The uptake amount of all different-sized LDHs is not affected by the presence of serum, indicating that LDH nanoparticles actively select their own entry and do not enter cells by using the uptake route of serum after adsorption onto serum surface. Figure 9 shows the cellular uptake and retention behavior of LDHs as a function of incubation time, with particle size dependency. The 50 nm LDH is observed to be rapidly endocytozed and its maximum internalization occurs in the very early stage of incubation time, and further more maintains the high concentration during all the incubation period (Figure 9A). The quite similar uptake behavior can be seen for the 100 and 200 nm particles. No significant increase in cellular uptake could be seen for the 350 nm LDH upon incubation time. In order to evaluate the particle retention capacity, the cells are treated with LDHs, washed with PBS to remove any remaining LDH and further incubated in fresh medium without LDHs. All different-sized LDHs except 50 nm are well retained in the cells during the entire incubation time (Figure 9B). But, the intracellular level of the 50 nm LDH decreases dramatically for the first 8 h, suggesting their possible efflux into the extracellular environment. The 50 nm LDH with a large surface area and high positive surface charge may be more rapidly decomposed than the larger ones in acidic intracellular condition, leading to easy exocytosis.

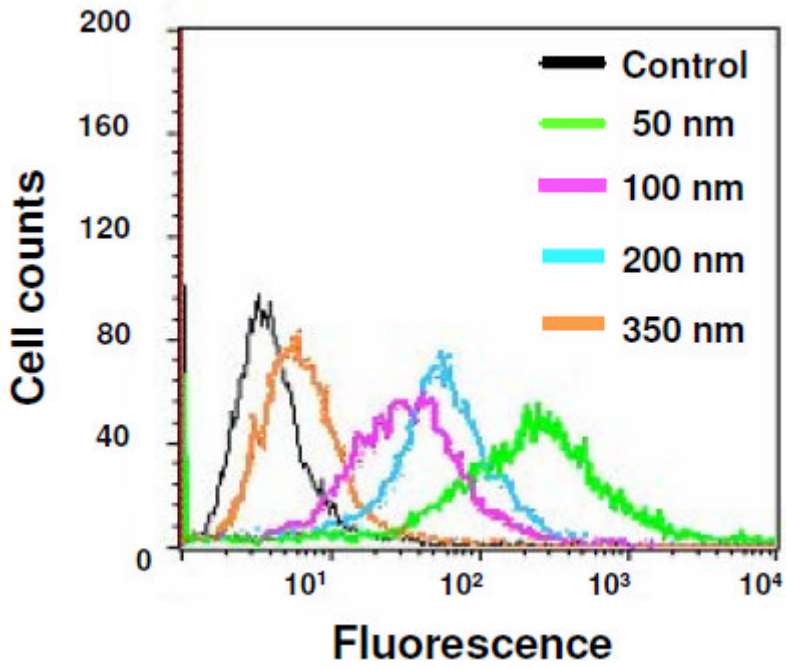

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Figure 8. Cellular uptake of LDH-FITC as a function of LDH size in MNNG/HOS cells.

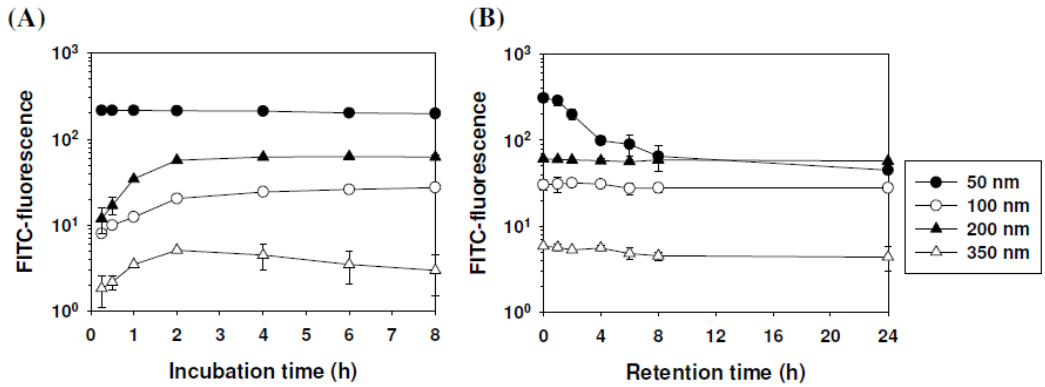

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Figure 9. Cellular uptake and retention kinetics of four different-sized LDHs: cellular uptake (A) and retention (B) of LDHs as a function of incubation time.

In order to determine the size effects on endocytosis pathways of LDH, the uptake modulation is evaluated by pre-incubating the cells with different endocytosis inhibitors, followed by treatment with FITC-LDHs. As well known, chloropromazine (CPZ) prevents the assembly of coated pits at the plasma membrane.[81] Potassium depletion and hypertonic medium also reduce membrane-associated ***clathrin*** lattices.[82] Filipin inhibits caveolin-1 association and disrupts caveolar structural integrity.[83] Genistein, an inhibitor of tyrosine

kinases, inhibits caveolae/raft-mediated endocytosis.[58] ***Macropinocytosis*** can also be inhibited by ***amiloride*** and ***amiloride*** analogues, which inhibit Na^+/H^+ exchange.[84] The internalization amount of LDH with each size of 50, 100 and 200 nm is suppressed about 80 – 85 % when the clathrin-mediated endocytosis is blocked by CPZ or potassium depletion (Figure 10). However, the uptake of the 350 nm LDH decreases about 50 – 60% by all the endocytosis inhibitors used, suggesting non-specific cell entry. This result clearly indicates that LDHs in the size range of 50 to 200 nm select clathrin-mediated internalization process, which is also correlated with high cellular uptake of these particle sizes. As mentioned earlier, the size of clathrin-coated vesicle is about 120 nm, but recruitment of internalization machinery for clathrin may adapt and accommodate its size that can extend beyond the actual size of a clathrin domain, displaying an upper limit of approximately 200 nm.

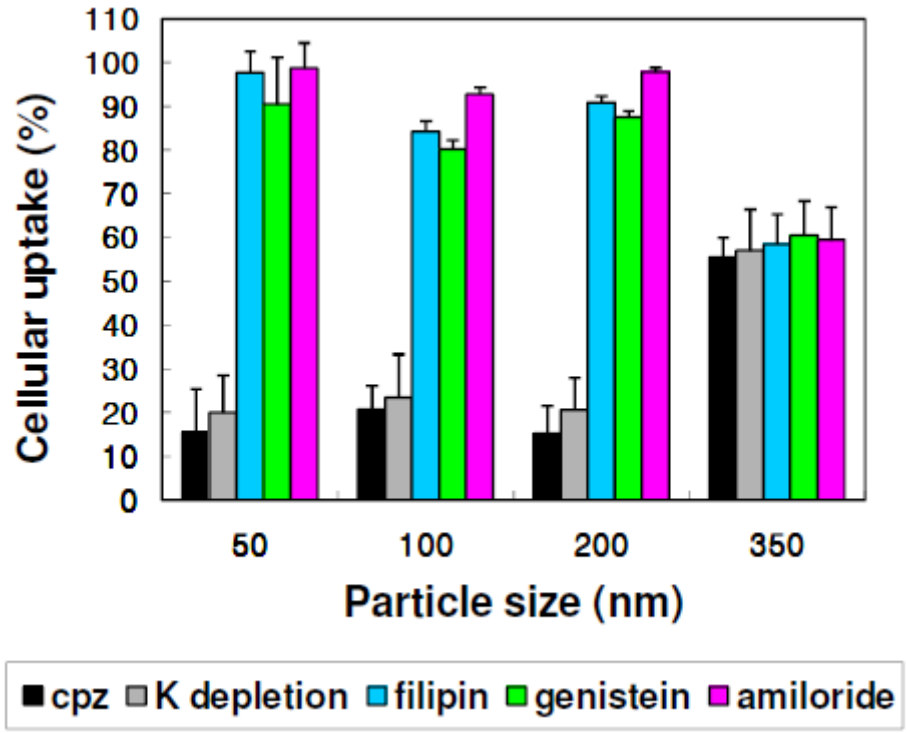

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Figure 10. Effects of endocytic inhibitors on the internalization of LDH-FITC. Cellular uptake (%) was calculated by comparison with that in the absence of inhibitor (100%) for each particles.

5.2. Surface Modification: Folate Conjugates

Recently, many drug delivery vectors including nanoparticle-based delivery systems have been developed in order to provide both target-specific delivery and efficient cellular uptake of anticancer agents in malignant tumor tissues. In addition, the rapid progress in nanotechnology enables to design and manipulate nano-structured materials for surface modification and target-oriented ligand conjugation. In our laboratory, we have devoted

ourselves to improve surface properties of LDHs by controlling the size, surface coating and ligand conjugations such as FITC or RITC for intracellular tracing.[47, 48, 85] Among various cancer cell targeting moieties, folic acid (FA), also kwown as vitamin B12, have attracted much interest, since it has high affinity (K_D ~ 100 pM) for folate receptors (FRs) often overexpressed in many human cancers including ovary, cervix, breast, lung, kidney, head, neck, colon cancer and etc.[29, 30, 86, 87]

The pristine LDH with a size of 100 ± 25 nm is prepared and then modified for its surface conjugation with FA.[47] Also, both LDH and LDH-FA are labeled with green fluorescent probe FITC (LDH-FITC or LDH-FA-FITC) for quantitative analysis of cellular uptake by means of flow cytometry as well as fluorescence microscopic visualization. The target-specific delivery efficiency of FA grafted LDH (LDH-FA) is evaluated in FR-overexpressing KB cells and FR-deficient A549 cells, respectively. KB cells are maintained in RPMI medium without any folate to intensively induce FR-overexpression, while FR-deficient A549 cells are obtained by culturing the cells in the presence of excess folate. It is worthy to mention here that the overall structure and morphology of LDH-FA are not changed but almost identical to the pristine LDH after FA grafting reaction. According to the fluorescence microscopic observation, both LDH-FITC and LDH-FA-FITC (50 μg/ml) are indeed internalized into cells and principally found in the cytoplasm after incubation for 2 h, but with a different manner at the periphery of the plasma membrane (Figure 11A); LDH-FITCs are largely distributed throughout the cytosol in punctuated forms and in part, the cell membrane as well, while LDH-FA-FITCs were highly localized at the periphery of the plasma membrane where FRs might be actively expressed.

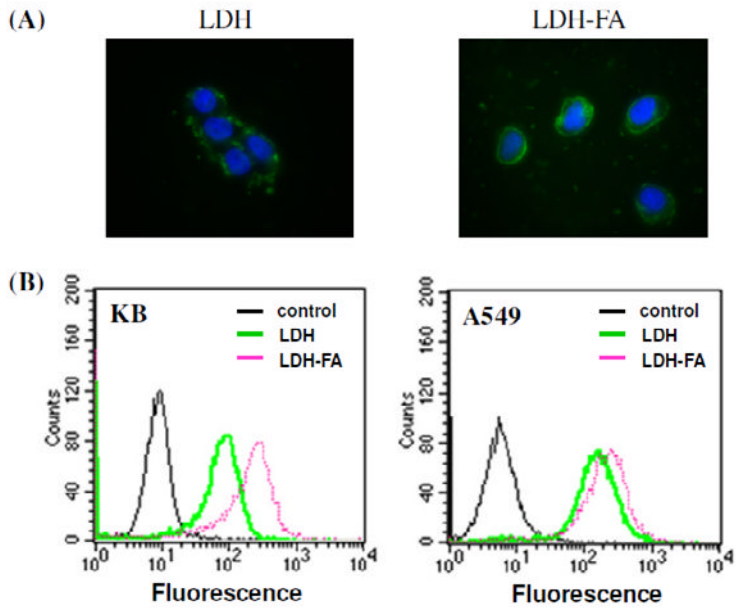

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Figure 11. (A) Fluorescence microscopic images of KB cells treated with either FITC-labeled LDH or FITC-labeled LDH-FA. (B) Fluorescence activated cell sorter (FACS) analysis of LDH and LDH-FA treated (a) KB and (b) A549 cells.

The specific selectivity of LDH-FA-FITC to FR-overexpressing cell line is also confirmed by quantitatively analyzing its uptake in comparison with that in A549 cells by means of flow cytometry. The cellular uptake of LDH-FA-FITC in KB cells increases approximately three folds compared to that of LDH-FITC after incubation for 2 h (Figure 11B). On the other hand, no significant difference in cellular uptake occurs between LDH-FITC and LDH-FA-FITC in A549 cells. This result clearly demonstrates that FA is structurally well conjugated to the surface of LDH, which leads to selective FA-FR interactions in FR-overexpressed cells.

Specific interaction between LDH-FA and FR on the plasma membrane which enhances cellular uptake of LDH-FA is also confirmed by pre-incubating the cells with excess amount of FA (125 μM) and subsequently saturating FR receptor binding sites. The uptake amount of LDH is not significantly affected by pre-incubation with excess FA both in KB cells and A549 cells (Figure 12). Interestingly, FA pre-incubation considerably reduces the cellular uptake of LDH-FA in KB cells, while not in A549 cells, indicating that the internalization of LDH-FA is processed by FR-mediated endocytosis. On the basis of above results, it is concluded that LDH-FA is specifically selective to FR only in FR-overexpressing cancer cells.

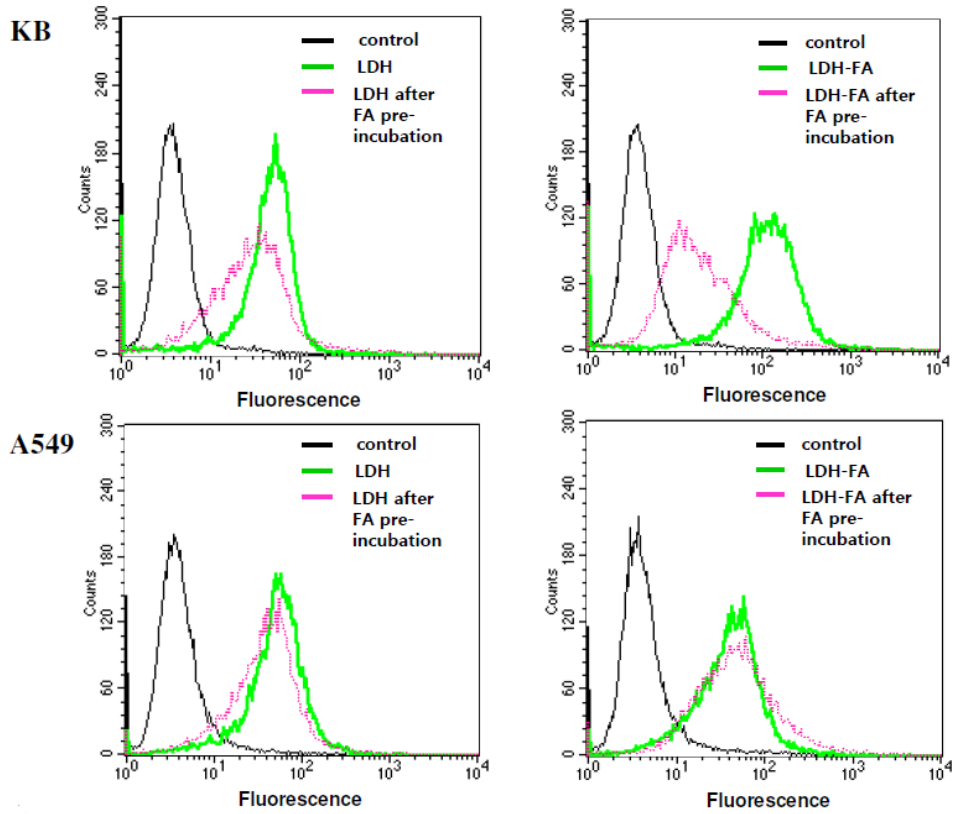

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Figure 12. Fluorescence activated cell sorter (FACS) analysis of LDH and LDH-FA treated KB and A549 cells with and without FA pre-incubation. The cells were pre-incubated with 125 μM of free FA before LDH and LDH-FA treatment.

It is noticeable that the pristine LDH can largely enter both cell lines without ligand conjugation. As we mentioned earlier, the cell entry of LDH nanoparticles is mainly related to the most common endocytic pathway, clathrin-mediated endocytosis. Therefore, the surface modification of LDH with cancer cell target-specific ligand FA leads to its selective uptake to FR-overexpressing cells, whereas, LDH without ligand or LDH-FA in FR-deficient A549 cells possess clathin-mediated endocytic property, which still remains to be effective in terms of cell permeation.

Drug delivery efficiency of LDH-FA is also evaluated by employing MTX encapsulated with two different LDH carriers, with or without FA ligands (MTX/LDH-FA or MTX/LDH), in KB cells and A549 cells (Figure 13). The internalized concentration of MTX in KB cells and A549 cells is quantitatively analyzed by HPLC after treatment with 50 μg/ml of either MTX/LDH or MTX/LDH-FA. As expected, the intracellular MTX level increases about 1.5 folds when delivered by MTX/LDH-FA compared with that transported by MTX/LDH in KB cells. On the other hand, no increase in MTX concentration could be observed in A549 cells, when delivered by MTX/LDH-FA. This is a clear evidence that the selective drug delivery to FR-overexpressing cells can be achieved by using LDH-FA nanovehicles, which finally enhances drug uptake.

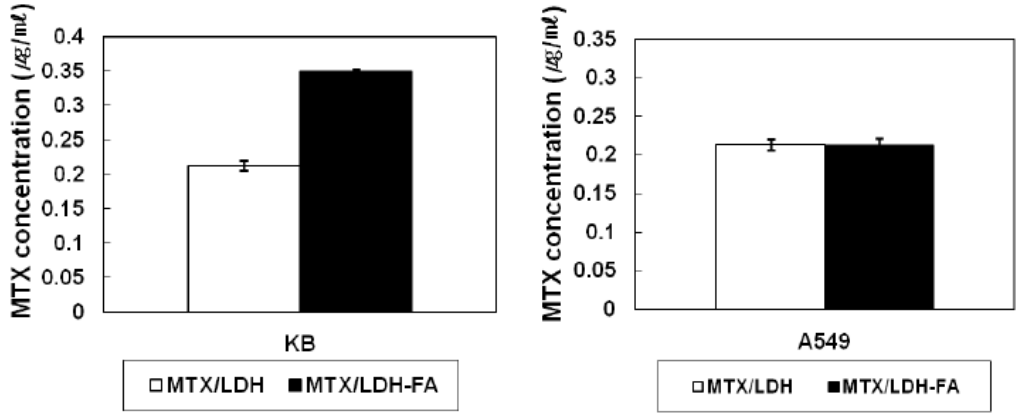

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Figure 13. Cellular MTX level in MTX/LDH and MTX/LDH-FA treated KB and A549 cells.

Anticancer effects of MTX/LDH-FA are also evaluated to elucidate the correlation between enhanced drug uptake and drug efficacy. As shown in Figure 14, MTX/LDH-FA suppresses more effectively the cell proliferation of KB cells compared with MTX/LDH. But, very similar anticancer activity of MTX/LDH-FA can be seen in A549 cells when compared with MTX/LDH. This result indicates that the enhanced uptake of MTX with MTX/LDH-FA is directly related to an increased drug efficacy in KB cells. It is worthy to note here that LDH-FA nanovehicle itself does not influence on cell proliferation as clearly demonstrated on both KB cells and A549 cells, suggesting that the present LDH-FA is not cytotoxic even after the surface modification and eventually not influencing on cell proliferation. Therefore, we conclude that drug encapsulated by LDH-FA nanocarriers can be effectively and selectively delivered to the target-specific FR-overexpressing cancer cells, resulting from specific FA-FR interaction.

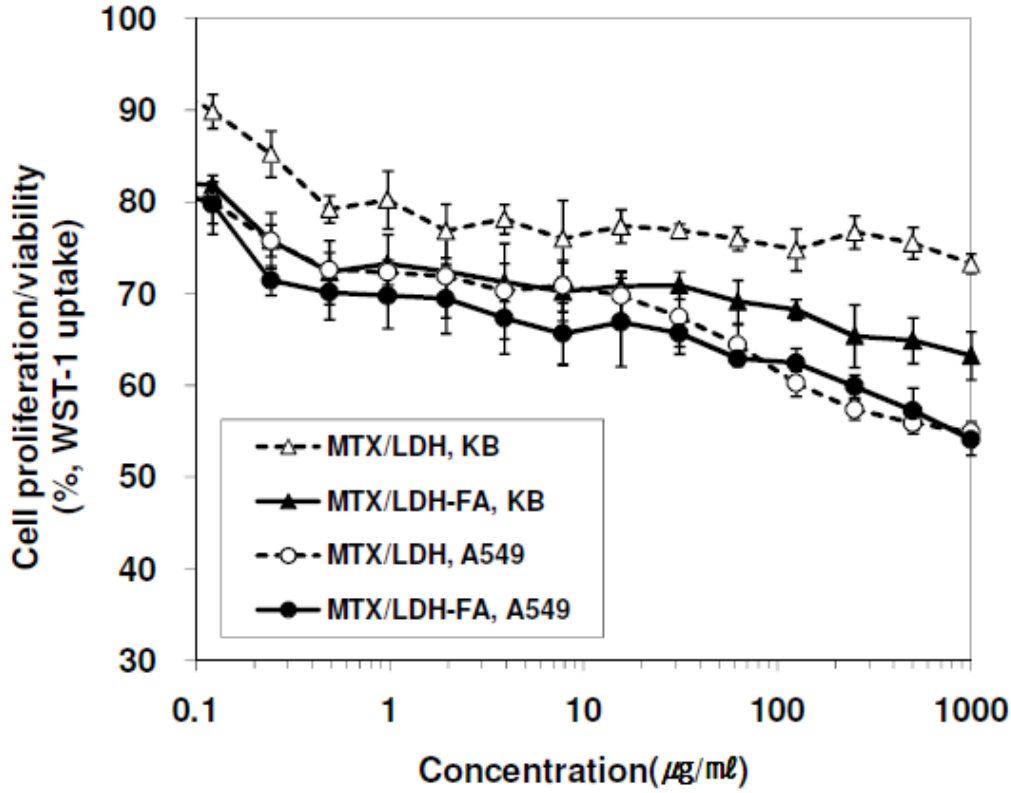

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Figure 14. Cell proliferation/viability of MTX/LDH and MTX/LDH-FA treated KB and A549 cells.

6. CONCLUSION

In this chapter, we describe the cellular uptake mechanism of LDH nanocarriers and try to demonstrate them as delivery systems for drug or bioactive molecules. The uptake of the present LDH nanoparticles is surely mediated by clathrin-coated process, the most common endocytic pathway in mammalian cells. The particle size itself plays an important role in the internalization of LDH via clathrin-mediated endocytosis, demonstrating its efficient cellular uptake in the size range of 50 to 200 nm. With MTX intercalated LDH as a model drug delivery system, the uptake mechanism of LDH has been determined, and found to be strongly correlated with anticancer effects, due to an efficient permeation capacity of LDH nanocarriers. It is also confirmed that the surface modified LDH with FA ligand can efficiently and selectively deliver the drug to target-specific FR-overexressing cancer cells, indicating that target specific ligand-receptor interaction can be easily achieved by LDH delivery carrier. Understanding the interaction mechanism of delivery nanocarriers with cells will provide a new perspective for the design of DDS with maximized and targeted delivery ability.

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Chapter 6

LIM KINASE 1/COFILIN SIGNALING PATHWAY PLAYS A PIVOTAL ROLE IN REGULATING EGF RECEPTOR ENDOCYTOSIS IN INVASIVE HUMAN BREAST CANCER CELLS

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ABSTRACT

The small GTPase Rho and Rho-associated protein kinase (ROCK) signaling pathway has been demonstrated to be one of the major pathways involved in tumor invasion through reorganization of actin cytoskeleton. It was shown previously that an expression of constitutively active form of RhoA or of ROCK in rat hepatoma cells considerably promoted invasive ability of these cells in vitro and in vivo, and enhanced phosphorylation level of myosin light chain MLC20, thereby, indicating that Rho-ROCK pathway mediates tumor cell motility and invasion. ROCK can phosphorylate and activate LIM kinase 1 (LIMK1), which leads to phosphorylation of cofilin. LIMK 1 is known to play a critical role in actin cytoskeletal remodeling by linking the signal from the Rho family of small GTPases to the change in cofilin activity, thereby, indicating an important role for Rho-ROCK-LIMK1-cofilin signaling in tumor cell invasion through regulating actin dynamics. We reported previously that overexpression of LIMK1

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resulted in a marked retardation of EGF receptor endocytosis in low-invasive human breast cancer cell MCF-7. Thereby, we postulate that LIMK 1 signaling plays an important role in the regulation of ligand-induced endocytosis of EGF receptor in tumor cells by reorganizing and influencing actin-filament dynamics. In the present study, we further assessed the effect of wild-type LIMK1, a kinase-deficient dominant negative mutant of LIMK1 (DN-LIMK1) and an active, unphosphorylatable cofilin mutant (S3A cofilin) on internalization of EGF-EGF receptor in MDA-MB-231, a highly invasive human breast cancer cell line. We demonstrate here that a marked delay in the receptor-mediated internalization of Texas red-labeled EGF was observed in the wild-type LIMK1 transfectants, and that most of the internalized EGF staining was accumulated within transferrin receptor-positive early endosomes even after 30 min internalization. In contrast, the expression of dominant-negative LIMK1 mutant rescued the efficient endocytosis of Texas red-EGF, and large amounts of Texas red-EGF staining already reached LIMPII-positive late endosomes/lysosomes after 15 min internalization. We further analyzed the effect of S3A cofilin mutant on EGF receptor endocytosis, and found an efficient delivery of Texas red-EGF into late endosomes/lysosomes at 15-30 min after internalization. Taken together, our novel findings imply that LIMK1-cofilin signaling indeed plays a pivotal role in the regulation of EGF receptor endocytosis via the early/late endocytic pathway in invasive tumor cells.

Keywords: LIM kinase 1; EGF receptor (EGFR); Cofilin; Lysosomes; Endocytosis; Tumor invasion.

INTRODUCTION

The small GTPase Rho signaling pathway has been demonstrated to be one of the major pathways involved in tumor invasion through reorganization of actin cytoskeleton (Itoh et al., 1999; Yoshioka et al., 1998, 1999, 2003). An expression of constitutively active form (Val14) of RhoA in rat hepatoma cells (MM1) considerably promoted invasive ability of these cells in vitro and in vivo, and enhanced phosphorylation level of myosin light chain MLC20 (Yoshioka et al., 1998, 1999). Among the Rho targets are the family members of the Rho-associated serine-threonine protein kinases (ROCK) (Amano et al., 1996; Ishizaki et al, 1997; Matsui et al., 1996), and ROCK also participates in cell-to-substrate adhesions, stress-fiber formation, and stimulation of actomyosin-based cellular contractility. ROCK, like Rho, has been demonstrated to be involved in tumor invasion (Itoh et al., 1999). Further, we previously demonstrated that overexpression of RhoA and ROCK in human epithelial cell causes an aberrant redistribution of lysosomes (Nishimura et al., 2000, 2002, 2003).

LIMK1 regulates actin dynamics by inhibiting the activity of the actin depolymerizing factor cofilin (Arber et al., 1998; Yang et al., 1998). It has been demonstrated that ROCK can phosphorylate and activate LIM kinase 1 and 2 (LIMK1 and LIMK2) (Amano et al., 2001; Ohashi et al., 2000) as well as myosin light chain 20 (MLC20) (Amano et al., 1996). These findings imply that Rho-ROCK signaling pathway activates LIMK1, which leads to phosphorylation of cofilin. Cofilin activity is reversely regulated by phosphorylation and dephosphorylation at Ser-3, with the phosphorylated form being inactive (Agnew et al., 1995; Moriyama et al., 1996). Phosphorylated cofilin shows reduced actin binding capability, thus favoring net actin polymerization. Therefore, LIMKs play a critical role in actin cytoskeletal

remodeling by linking the signal from the Rho family of small GTPases to the change in cofilin activity. Recent study showed an important role for Rho-ROCK-LIMK1 signaling in tumor cell invasion through regulating actin dynamics (Itoh et al., 1999; Yoshioka et al., 2003).

We also demonstrated that the overexpression of LIMK1 in less invasive cell lines, MCF-7, resulted in a marked retardation of the internalization of the receptor-mediated endocytic tracer, Texas red-labeled EGF, and that internalized EGF was accumulated in transferrin receptor-positive early endosomes instead of being transported to late endosomes (Nishimura et al., 2004). Thereby, we postulate that LIMK1 signaling pathway plays an important role in the regulation of ligand-induced EGFR vesicle traffic through the endocytic pathway in invasive tumor cells by reorganizing and influencing actin-filament dynamics. At present, the outline of the endocytic pathway appears to be established, but the molecular mechanisms underlying the sorting and trafficking events in endosomes are still remains to be seen. Hence, the identification and characterization of each regulatory component in the endocytic pathway are of importance for the further clarification of its machinery. In the present study, we have further assessed the possible role of LIMK1 in the endocytic pathway of ligand-induced EGFR by employing a kinase-deficient dominant negative form of LIMK1 (DN-LIMK1) or an active, unphosphorylatable form of S3A cofilin mutant in MDA-MB-231, a highly invasive human breast cancer cell line. We here demonstrate that EGF-EGFR traffic out of early endosomes is impaired by the expression of LIMK1, whereas the transfection of kinase-deficient LIMK1 mutant or S3A cofilin mutant rescues the efficient endocytosis of ligand-induced EGFR in MDA-MB-231 cells. Taken together, we postulate that kinase activity of LIMK1 may coordinate vesicular traffic of EGFR out of early endosomes.

MATERIALS AND METHODS

Materials

Texas red-labeled transferrin, Texas red-labeled EGF, Texas red-labeled phalloidin, and SlowFade anti-fade reagent were purchased from Molecular Probes (Eugene, OR, USA). MDA-MB-231 human breast cancer cells were cultured as described (Hiraga et al, 2001). The molecular weight mass markers for immunoblotting were from Invitrogen (Carlsbad, CA, USA). Other chemicals were of reagent grade and were obtained from commercial sources.

Transfections

Myc-tagged DNA constructs encoding mouse LIMK1, LIMK1 (-)[LIMK1-short, which lacks 20 amino acids in the catalytic domain; dominant negative LIMK1(DN-LIMK1)], and unphosphorylatable S3A cofilin mutant were described elsewhere (Arber et al., 1998). Stable cell lines were generated by transfection of constructs or empty vector by using Lipofectamine Plus reagent (Invitrogen), and selected in the presence of 800 μ g/ml G418 for 4 weeks and cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 10% fetal calf serum (FCS; Equitech-bio, TX, USA).

Antibodies

Alexa 488-labeled goat anti-mouse, goat anti-rabbit secondary antibodies, Texas red-labeled human transferrin, and Texas red-labeled EGF were obtained from Molecular Probes (Eugene, OR, USA). Antisera were raised in rabbits (New Zealand white male) against the mature form of rat liver lysosomal cathepsin D (Nishimura et al., 1988) and the native form of LIMPII/LGP85 (Okazaki et al., 1992) as described previously. Each specific IgG was affinity-purified by protein A Sepharose CL-4B (Amersham Pharmacia Biotech, Piscataway, NJ, USA), followed by immunoaffinity chromatography using antigen-conjugated Sepharose 4B. Rat anti-LIMK1 monoclonal antibody was reported previously (Foletta et al., 2004). Mouse monoclonal antibodies to early endosome autoantigen 1 (EEA1) and syntaxin 6 were purchased from BD Biosciences (San Jose, CA, USA). Anti-transferrin receptor monoclonal antibody was obtained from Immunotech (France). Mouse monoclonal anti-human lysosome-associated membrane protein-1 (LAMP-1) was purchased from the Developmental Studies Hybridoma Bank maintained by the University of Iowa (Iowa City, IA, USA). A mouse monoclonal anti-EGFR was obtained from BD Biosciences (San Jose, CA, USA) and DakoCytomation (Denmark).

Immunofluorescence Microscopy

Immunofluorescence microscopy was previously described (Nishimura et al., 2000, 2002, 2003, 2004, 2006). The cells were grown for 2 days on glass coverslips in 6-well plates in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, and were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS), pH 7.4, permeabilized in PBS containing 0.1 % saponin. The mock, LIMK1, DN-LIMK1, or S3A cofilin transfectants were incubated with specific primary antibodies (rabbit anti-cathepsin D and anti-LIMPII IgGs, mouse anti-LAMP1 mAb, mouse anti-EGFR mAb, mouse anti-EEA1 mAb, mouse anti-syntaxin 6 mAb, or mouse anti-transferrin receptor mAb), for 1 hour, followed by washes with PBS containing 0.1 % saponin and incubation for 1 hour with the secondary antibodies at 20μg/ml. In order to label early endosomes, cells were incubated for 20 min with Dulbecco's modified Eagle's medium containing 10% fetal calf serum and Texas red-conjugated transferrin. Then cells were fixed and stained for EGFR or syntaxin 6. To follow the endocytic pathway and determine the intracellular fate of internalized labeled ligand, the uptake of Texas red-conjugated-EGF for 5min, 15min, and 30min at 37^{o}C by the cells was measured. In order to minimize the contribution of recycling and/or lysosomal degradation of internalized EGFR, we quantified the Texas red-EGF uptake for relatively brief time periods (up to 30 min). The distribution of the labeled proteins was analyzed by confocal immunofluorescence microscopy of the fixed cells. Slides were observed on a Zeiss LSM 510 META confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany), equipped with krypton/argon laser sources. To obtain 3D-imaging data sets or to generate optical sections of the cells by confocal laser scanning microscopy, 30 optical sections (each X-Y section) along the Z-axis were acquired as described previously (Nishimura et al., 2004). A significant overlapped area in the 2D-histogram was selected by using the colocalized area button, and thus selected colocalization appeared as white pseudo-color image in the superimposed data, and contrast levels of the images were adjusted using Adobe Photoshop

6.0 software (Adobe Co.) on MacBook G4 computers (Apple, Tokyo, Japan). For quantitation of colocalization between Texas red-transferrin and EGFR or between Texas red -EGF and LIMPII, merged images as yellow color or white color were quantitated and presented as the percentage of total amounts of EGFR- or LIMPII-positive vesicles per cell.

Immunoblotting Analysis

Cell fractionation of the MDA-MB-231 transfectants was carried out as described previously (Yoshioka et al. 1999). The cells were grown to subconfluency (60-70%) in DMEM with 10% FCS, and the medium was then replaced with serum-free DMEM containing 0.5% fatty acid-free BSA for 24 h. Then the cells (2×10^6 cells) were lysed by freeze-thawing in ice-cold 300μl of lysis buffer [50mM HEPES (pH 7.5), 50mM NaCl, 1mM $MgCl_2$, 2mM EDTA, 10mM NaF, 1mM DTT, 1mM phenylmethylsulphonylfluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin] and centrifuged at 100,000 x *g* for 30 min at 4^0C and the supernatant was collected as the cytosol fraction. The pellet was resuspended, homogenized in 300 μl of lysis buffer containing 2% Triton X-114, and then centrifuged at 800 x *g* for 10 min. The supernatant was collected and is referred to here as the membrane fraction. The cytosol fraction and the membrane fraction were separated by SDS-PAGE followed by immunoblotting analysis. In order to detect LIMK1 or EGFR protein in each cell transfectants, cells (5 x 10^4) were lysed, separated by 7% SDS-PAGE for LIMK1 or by 10% SDS-PAGE for EGFR and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA, USA) with semi-dry method. The blot was probed with rat anti-LIMK1 mAb (1:3000), anti-Myc mAb (1:3000; Santa Cruz Biotechnology, CA, USA), anti-RhoGDI mAb (1:2500; BD Biosciences), anti-EGFR mAb (1:1000; BD Biosciences), or with anti-β-actin mAb (1:500; Chemicon, Temecula, CA, USA). The secondary antibodies were alkaline phosphatase-conjugated anti-rat (1:2500), anti-mouse (1:7500) or anti-rabbit (1:7500) IgG (Promega, Japan). For estimation of phospho-cofilin levels, each transfectant (3 x 10^4) was starved in medium containing 0.1%BSA for 24 h before harvesting and separated on 12.5%SDS-PAGE followed by immunoblot analysis. The blot was probed with rabbit anti-phospho-cofilin (1:1,000; Cell Signaling Technology, Beverly, MA, USA), and rabbit anti-cofilin (1:200; Cell Signaling Technology) polyclonal antibodies, followed by goat anti-rabbit IgG alkaline phosphatase conjugate (Promega, 1; 7,500). The membrane was scanned with GT 9500 flat scanner (Epson, Tokyo, Japan) and analyzed with NIH image software using a MacBook G4 computer (Apple, Tokyo, Japan).

RESULTS

LIMK1 Expression and Activity are Altered in the MDA-MB-231 Transfectants

The MDA-MB-231 cell line stably expressing mouse myc-tagged wild type (wt) LIMK1 or myc-tagged DN-LIMK1 were prepared and examined the level of endogenous and overexpressed LIMK1 proteins by immunoblotting analysis. The overexpressed mycLIMK1 protein migrates as a single band with mobility slower than that of endogenous LIMK1 with an apparent molecular weight of 66.5 kDa, and the expression level of the overexpressed wtLIMK1 or DN-LIMK1 was 7.73-fold or 6.0-fold higher than the endogenous protein, respectively (data not shown). We reported previously that MDA-MB-231 clones expressing high levels of LIMK1 (14~20-fold compared to the endogenous protein) present an increased invasiveness in the Matrigel invasion assay, while clones expressing high levels of DN-LIMK1 (3~9-fold) exhibited lower invasiveness, indicating that several-fold increase of LIMK1 in the MDA-MB-231 cells alter their invasiveness (Yoshioka et al., 2003).

Next, each transfectants were subjected to cell fractionation experiments, and then the cytosolic and membrane fractions were analyzed with SDS-PAGE followed by immunoblotting. In the mock- or DN-LIMK1 transfected cells, most LIMK1 was found in the cytosol fraction, in contrast, the transfection of LIMK1 cDNA into MDA-MB-231 cells caused an increased level of LIMK1 (10%) in the membrane fraction (Figure 1A). These results indicate that expression of LIMK1 cDNA enhances the translocation of expressed LIMK1 from the cytosol fraction to the membrane fraction. We also examined the levels of endogenous EGFR protein in each cell lines. Endogenous EGFR protein was revealed primarily as a 175-kDa single protein band localized in the membrane fraction in all of the cell lines tested (Figure 1A), and the protein level was not changed by the expression of LIMK1 or DN-LIMK1.

In order to further analyze whether changes in the LIMK1 expression in MDA-MB-231 cells affects the phosphorylation level of endogenous substrate, cofilin, we generated three MDA-MB-231 cell lines stably expressing myc-tagged wtLIMK1 or myc-tagged DN-LIMK1 proteins and the level of phosphorylation of cofilin was determined by immunoblotting (Figure 1B). The stable transfectants expressing wtLIMK1 showed 1.3- to 1.6-fold increase in the level of phosphorylated-cofilin as compared with that of mock-transfectants (clone1, 1.32-fold; clone 2, 1.43-fold; clone3, 1.56-fold). In contrast, DN-LIMK1 transfectants showed a significant decrease (0.9- to 0.6-fold change) in the level of phosphorylated-cofilin as compared with that of mock-transfectants (clone1, 0.89-fold; clone2, 0.66-fold; clone3, 0.58-fold). Therefore, we demonstrate that the LIMK1 activity as judged by the level of phosphorylated-cofilin was positively correlated with the expression level of overexpressed wtLIMK1, and negatively correlated with the expression level of DN-LIMK1. We also estimated the level of phosphorylation of cofilin in the stable transfectants expressing wt-cofilin or S3A cofilin mutant by immunoblotting and probing with anti-phospho-cofilin antibody (Figure 1 C). The stable transfectant expressing wt-cofilin showed highly phosphorylated level of cofilin as compared to mock-transfectants. In contrast, the immunoreactive phosphorylated-S3A cofilin band was not detected on the immunoblot, therefore, confirming that S3A cofilin mutant is truly unphosphorylatable.

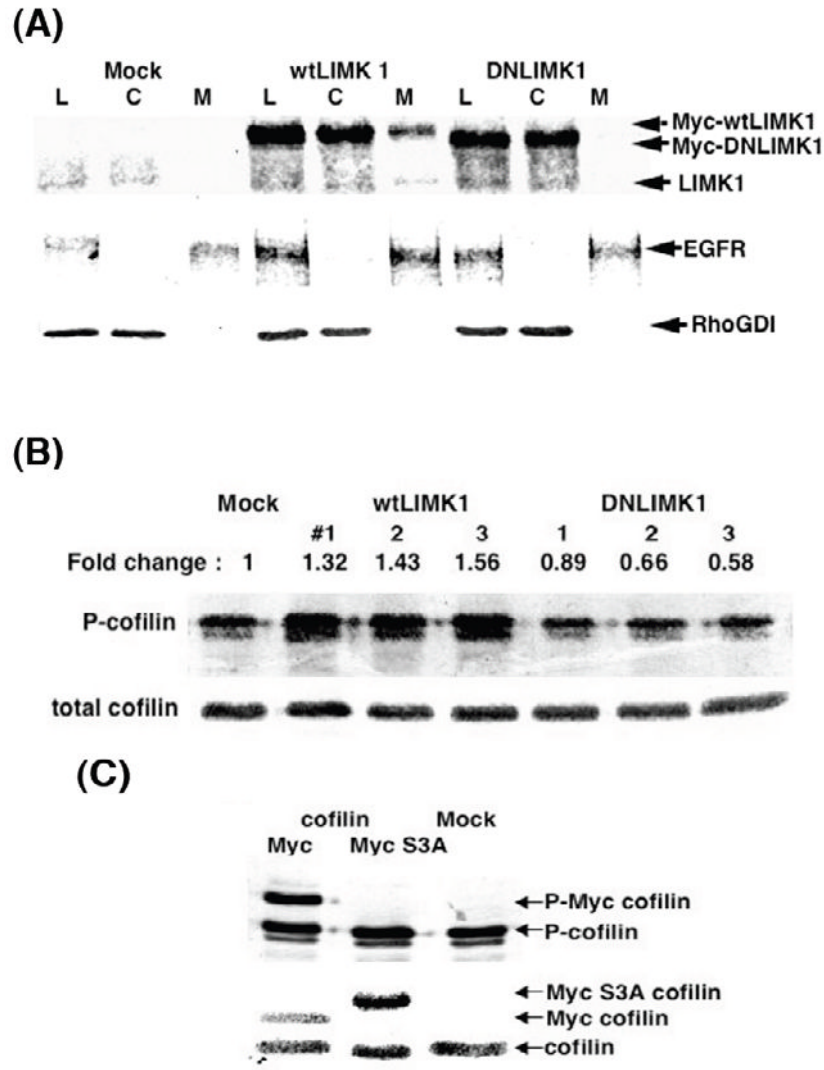


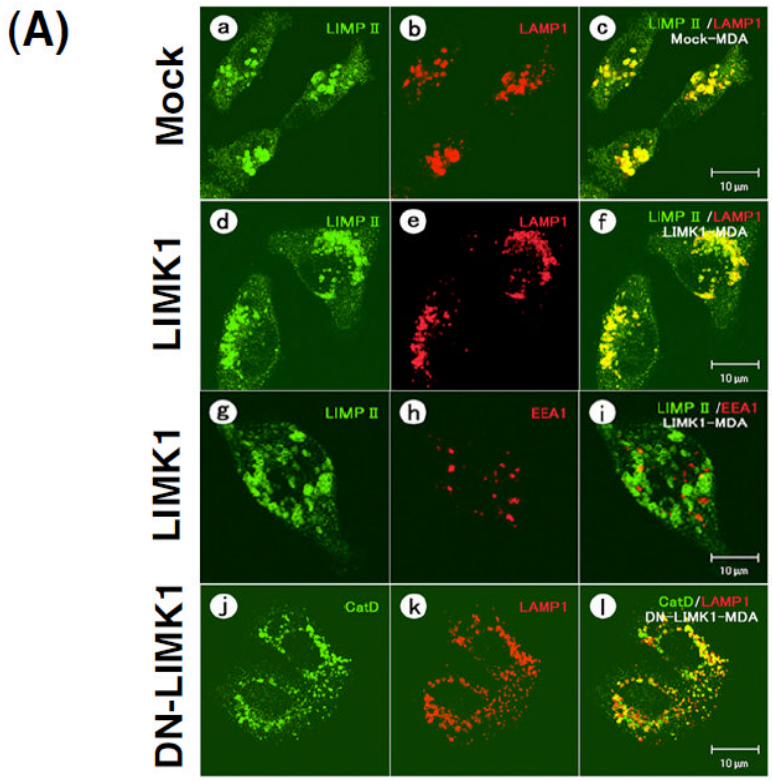
Figure 1 is the syntheses of data published previously by Nishimura et al. in 2006.

Figure 1. Immunoblot analysis of LIMK1, EGFR, or phosphorylated cofilin expression in mock-, wtLIMK1-, DN-LIMK1-transfectants. (A) Intracellular distribution of LIMK1, EGFR and RhoGDI in the mock-, wtLIMK1-, and DN-LIMK1-transfected MDA-MB-231 cells. The cell lysates (L) extracted from each transfectant were fractionated to separate the cytosolic fraction (C) and membrane fraction (M) as described in Materials and methods, and each fraction was subjected to SDS-PAGE followed by immunoblot analysis with anti-LIMK1 (upper panel), anti-EGFR (middle panel), or anti-RhoGDI (lower panel) antibody. Migration of the expressed tagged-LIMK1 or endogenous LIMK1 is indicated by arrows. (B) Immunoblot analysis of cell lysates prepared from mock-, wtLIMK1-, and DN-LIMK1 transfectants probed with anti-phospho-cofilin (upper panel) and anti-cofilin antibodies (lower panel). The fold-change in the level of phosphorylated cofilin is indicated above the upper panel. The data are representative of three independent experiments. (C) Immunoblot analysis of cell lysates prepared from mock-, tagged-cofilin-, or S3A tagged-cofilin-transfectants probed with anti-phospho-cofilin (upper panel) and anti-cofilin antibodies (lower panel). The positions of the expressed phosphorylated Myc-cofilin, endogenous phosphorylated cofilin, Myc-S3A cofilin, tagged cofilin, and endogenous cofilin are indicated by arrows, respectively.

EGFR is Localized in the Cathepsin D- and LIMPII-Positive Swollen Vacuolar Structures in the MDA-MB-231 Transfectants

In order to determine the intracellular localization of cathepsin D, LIMPII, or LAMP1 in the mock, LIMK1, or DN-LIMK1 transfectants, each transfectant was double-labeled with specific antibodies to LIMPII and LAMP1 or with specific antibodies to cathepsin D and LAMP1, respectively. We examined the intracellular distribution of lysosomes by using specific antibodies to lysosomal aspartic protease cathepsin D or LIMPII/LGP85 (Tabuchi et al., 2000). We found that large numbers of cathepsin D- or LIMPII-positive large swollen vesicles were detected mostly in the perinuclear region of the mock-, LIMK1, or DN-LIMK1-transfectants, and cathepsin D- and LIMPII-positive swollen vacuoles in all three transfectants were co-stained with LAMP1 antibody (Figure 2).

We next examined distribution of EEA1, an established marker for early endosomes (Mu et al., 1995), in LIMK1 transfectants. It was found that punctuate staining of EEA1 spread in the cytoplasm of the transfected cells, but LIMPII positive-large vacuoles were completely devoid of EEA1 staining (Figure 2A). Therefore, these LIMPII-positive vacuoles are truly characteristic of late endosomes/lysosomes. Furthermore, it was revealed that overexpression of DN-LMIK1 did not change the morphology of these swollen vacuoles. Based on our results, it is suggested that the large swollen vacuoles are not derived from the overexpression of LIMK1.



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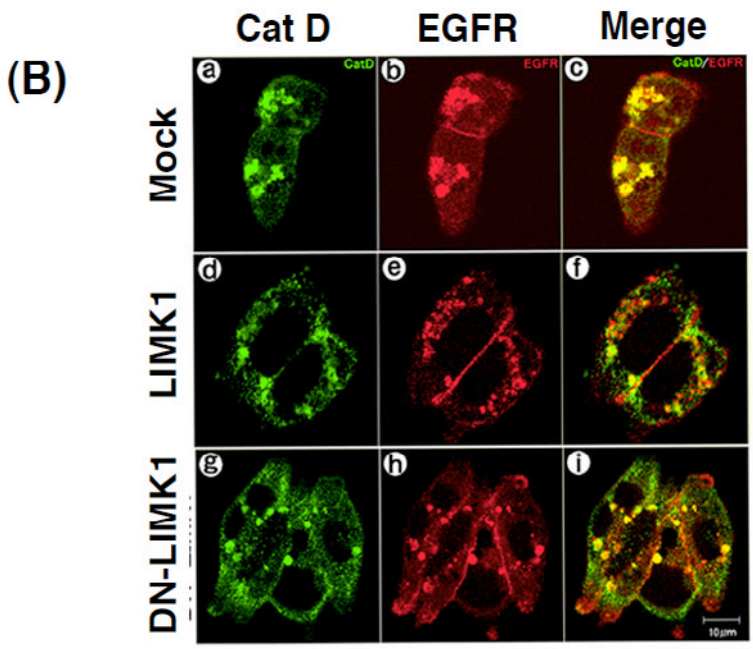


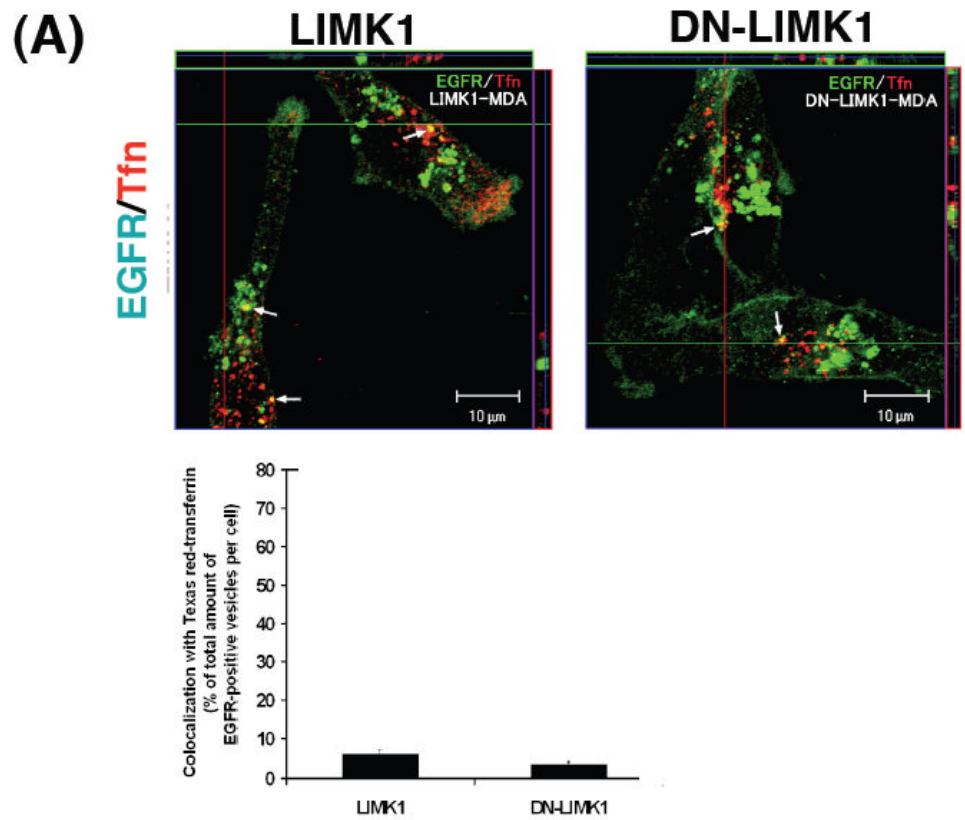
Figure 2 is the syntheses of data published previously by Nishimura et al. in 2006.

Figure 2. EGFR colocalizes with cathepsin D-positive swollen vacuolar compartments in MDA-MB-231 transfectants. In (A), Mock (a-c), LIMK1 transfectants (d-i) or DN-LIMK1 transfectants (j-l) were fixed and double-stained for LIMPII (a, d, and g), or cathepsin D (j) and LAMP1 (b, e, k), and EEA1 □h□ as described in Materials and methods. The right columns show the superimposed images of LIMPII (green) or cathepsin D (green) and each marker (red). In (B), Mock- (a-c), LIMK1- (d-f), or DN-LIMK1-transfected cells (g-i) were fixed and double-stained for cathepsin D (green) and EGFR (red) as described in Materials and Methods. Superimposed images of cathepsin D and EGFR are shown in c, f, and I in each transfectant. Bar, 10μm.

We also analyzed the cellular distribution of EGFR in the mock-, LIMK1-, or DN-LIMK1 transfectants, and the cells were co-stained for EGFR and cathepsin D or LIMPII, respectively. We found that EGFR staining was co-stained with cathepsin D- or LIMPII-positive large swollen vesicles (data not shown) in the perinuclear region of all transfectants, therefore, demonstrating that major distribution of EGFR was cathepsin D-, LIMPII-positive late endosomes/lysosomes (Figure 2B).

Next, in order to examine whether thus observed EGFR-positive vacuoles are a compartment that is accessible to endocytic marker, each transfectants expressing LIMK1 or DN-LIMK1 were allowed to internalize Texas red-labeled-transferrin for 20 min. In LIMK1- or DN-LIMK1-transfectants, Texas red-transferrin was efficiently internalized within the cell and accumulated within vesicular structures in the cytoplasm. It was shown that LIMK1 overexpression has no effect on the intracellular endocytic trafficking of transferrin during 20 min incubation. Importantly, we found that EGFR-positive vacuoles overlapped poorly with early endosomes labeled with the endocytosed Texas red-transferrin throughout the cytoplasm in the LIMK1 or DN-LIMK1 transfectants as demonstrated by quantitative analysis (Figure 3A). These results indicate that EGFR positive-large swollen vacuoles, colocalized with cathepsin D or LIMPII, are not accessible to endocytic markers, therefore, confirming that EGFR-positive vacuolar structures are originated from late endosomes/lysosomes. Our

previous data demonstrated that overexpression of LIMK1 in human breast cancer cells (MCF-7) result in the marked accumulation of internalized Texas red-EGF in early endosomes (Nishimura et al. 2004). Accordingly, we proposed that LIMK1 has some suppressive effect on the regulation of vesicle traffic out of early endosomes. This proposal gives rise to a possibility that recycling molecules such as syntaxin 6, reported to be involved in the recycling of transport vesicles from TGN to early endosomes (Bock et al., 1997), would be accumulated in the early endosomes where LIMK1 inhibits the vesicular traffic. To demonstrate this possibility, we examined distribution of the Texas red-transferrin, endocytic marker, by double labeling with anti-syntaxin 6 antibody in the LIMK1- or DN-LIMK-transfected cells. As shown in Figure 3B, large proportion of syntaxin 6 staining was accumulated in the small punctate structures which are clearly colocalized with the endocytosed Texas red-transferrin in the LIMK1 transfectants. We therefore assumed the accumulation of syntaxin 6 in the early endosomes (quantitated in Figure 3B). These results indicate that recycling pathway from early endosomes to TGN may be perturbed by the expression of LIMK1. In contrast, in the DN-LIMK1 transfectants, endocytosed Texas red-transferrin-positive early endosomes were clearly devoid of syntaxin 6 staining. These results indicate that overexpression of DN-LIMK1 normalize the recycling of syntaxin 6-positive transport vesicles from early endosomes to TGN. These results confirm that overexpression of LIMK1 has suppressive effects on some steps of the endosomal membrane traffic.



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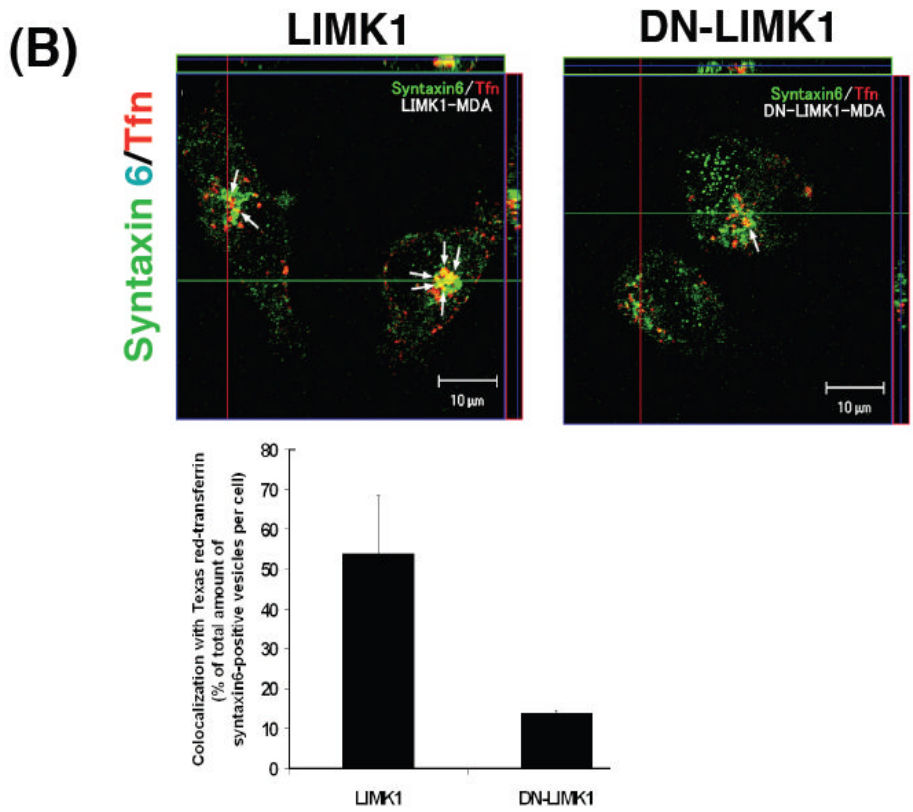

Figure 3 is the syntheses of data published previously by Nishimura et al. in 2006.

Figure 3. Recycling pathway for syntaxin 6 from early endosomes to TGN is perturbed by the expression of LIMK1 in MDA-MB-231 cells. In (A), LIMK1 or DN-LIMK1 transfectants were incubated for 20 min with Texas red-labeled transferrin (Tfn). The distribution of swollen vacuoles stained with anti-EGFR antibody (green) and the internalized Texas red-transferrin (red) was analyzed by confocal immunofluorescence microscopy after fixation of each transfectant. Superimposed images of EGFR and Texas red-transferrin for each orthographic section (at 0.35 μm from the cell bottom) of LIMK1 (left) and DN-LIMK1 (right) transfectants are shown. The merged confocal images as yellow color were quantitated and presented as the percentage of total amounts of EGFR-positive vesicles per cell. The error bar denotes SD. In (B), the LIMK1- or DN-LIMK1 transfectants were incubated for 20 min with Texas red-labeled transferrin (Tfn). Superimposed images of syntaxin 6-positive small punctate structures and the internalized Texas red-transferrin for each orthographic section of LIMK1 (left) and DN-LIMK1 (right) transfectants are shown. The white arrows indicate the colocalization of the vesicular structures with transferrin and syntaxin 6. The merged confocal images as yellow color were quantitated and presented as the percentage of total amounts of syntaxin 6-positive vesicles per cell. Bar, 10μm.

The Expression of Dominant-Negative LIMK1 or the S3A Cofilin Mutant Rescues Efficient Endocytosis of the Ligand-Induced EGFR in the MDA-MB-231 Cells

Next, we studied the intracellular fate of the internalized ligand-induced EGFR in early endosomes or late endosomes/lysosomes by double–immunolabeling confocal

immunofluorescence microscopy. In order to follow the endocytic pathway and determine the intracellular fate of ligand-induced EGFR, each transfectant was incubated at 37^0C for 5, 15, and 30 min with Texas red-EGF. The distribution of internalized Texas red-EGF, early endosomes stained with anti-transferrin receptor antibody, and late endosomes/lysosomes stained with anti-LIMPII antibody was then assessed by confocal immunofluorescence microscopy.

In the LIMK1-transfected MDA-MB-231 cells, after 15 min internalization, no EGF staining was found to be colocalized with the LIMPII-positive-late endosomes/lysosomes (Figure 4B). Furthermore, after 30 min internalization, the small vesicles that positively stained for the internalized EGF were found in the perinuclear region, however, EGF staining was clearly distinct from the LIMPII-positive lysosomes. These data indicate that the endocytic traffic of EGF-EGFR out of early endosomes toward late endosomes is considerably suppressed in the LIMK1-transfected cells. Because the internalized Texas red-EGF was revealed to be colocalized with transferrin receptor-positive early endosomes after 5 min internalization (Figure 4A,B), these data confirm that overexpression of LIMK1 does not suppress the early endocytic pathway of EGF-EGFR from plasma membrane to early endosomes.

We then analyzed the ligand-induced endocytosis of EGFR in the mock-transfected cells (Figure 4A). In the mock-transfected cells, only small amounts of EGF staining were found to be co-stained with LIMPII-positive late endosomes/lysosomes structures even after 30 min internalization, although considerable amounts of internalized EGF were colocalized with transferrin receptor-positive early endosomal structures after 5-30 min internalization. These results indicate that EGF-EGFR complex is not efficiently transported from early endosomes to late endosomal/lysosomes and is also accumulated in the early endosomal structures in the mock-transfected cells. These results appear to be in contrast to those in the mock-transfected MCF-7 cells in which efficient endocytosis of EGF-EGFR complexes proceeds normally and no accumulation of endocytosed EGFR in the early endosomes is not seen (Nishimura et al., 2004). Because endogenous LIMK1 expression level in MDA-MB-231 cells is reported to be several-fold higher as compared to that in MCF-7 cells (Yoshioka et al., 2003), we assumed that endogenous LIMK1 activity has some suppressive effect on the vesicular traffic of EGFR out of early endosomes in the MDA-MB-231 cells.

We next assessed the internalization of EGF by employing kinase-deficient dominant negative LIMK1 mutant. As expected, an efficient internalization of Texas red-EGF was observed in the dominant negative LIMK1 transfectants (Figure 5A). After 15 min internalization, the internalized Texas red-EGF was already found to be colocalized with the LIMPII-positive late endosomes/lysosomes, and after 30 min internalization, the endocytosed Texas red-EGF-positive small vesicles were revealed to be colocalized well with the LIMPII-positive late endosomes/lysosomes in the perinuclear region (Figure 5A). Computational 3D-image analysis of confocal immunofluorescence micrographs also demonstrates the significant overlap for the endocytosed EGF with LIMPII-positive late endosomes/lysosomes in the perinuclear region (data not shown).

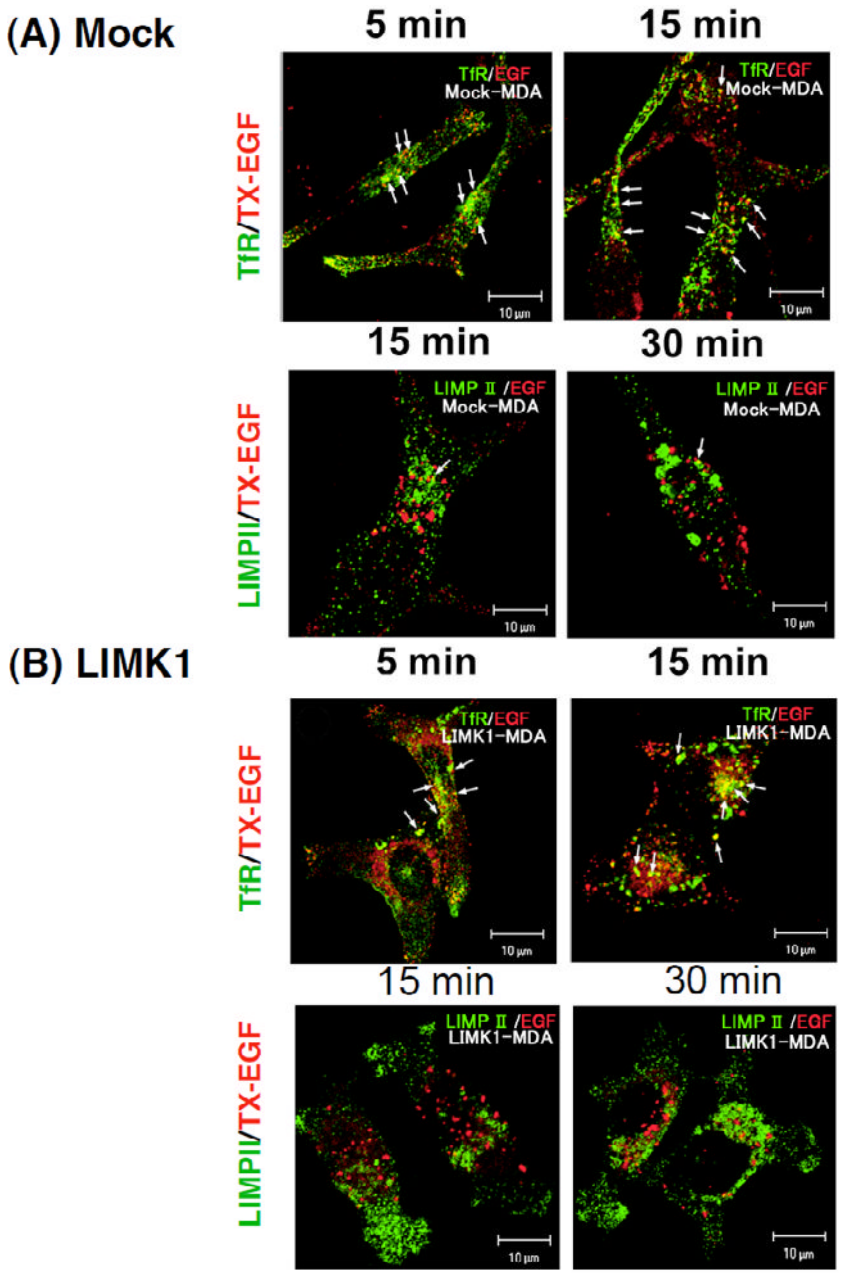

Figure 4 is the syntheses of data published previously by Nishimura et al. in 2006.

Figure 4. Endocytic delivery of ligand-induced EGFR from early endosomes to late endosomes is considerably suppressed in the Mock- or LIMK1-transfected cells. In A, Mock-transfected MDA-MB-231 cells were incubated at 37^0C with Texas red-EGF for 5 min, 15 min, or 30 min. The distribution of the internalized Texas red-EGF in early endosomes (stained with anti-transferrin receptor antibody) and the late endosomes/lysosomes (stained with anti-LIMPII antibody) was studied by confocal immunofluorescence microscopy after fixation of the cells, and superimposed images of EGF and transferrin receptor (TfR) or EGF and LIMPII are shown. In B, LIMK1 transfectants were treated as described above. The white arrows indicate the colocalization of the Texas red-EGF- and transferrin receptor-positive structures or Texas red-EGF- and LIMPII-positive structures, respectively. Bar, 10μm.

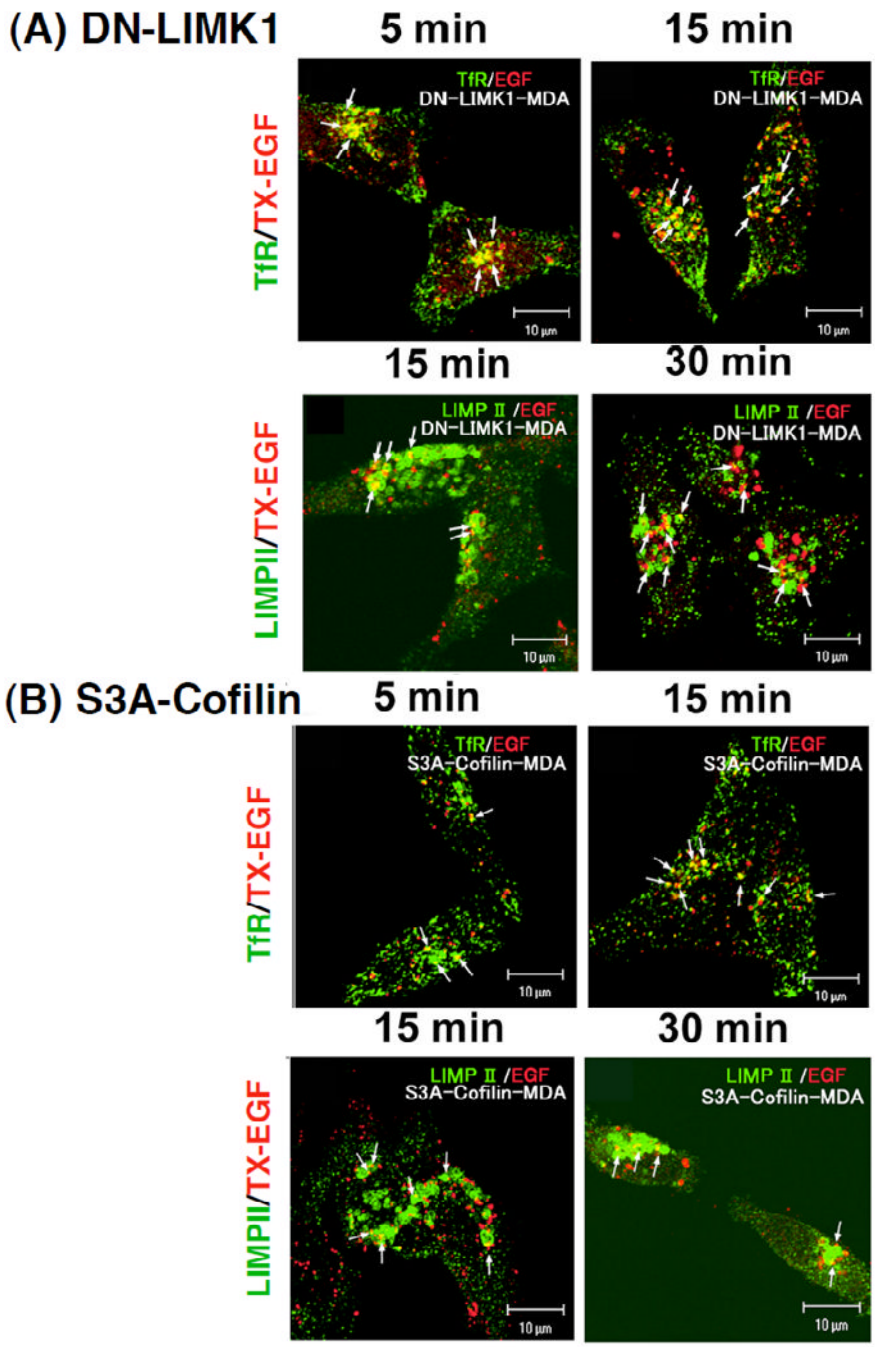

Figure 5 is the syntheses of data published previously by Nishimura et al. in 2006.

Figure 5. Expression of kinase-deficient dominant negative LIMK1 or unphosphorylatable mutant of cofilin rescues efficient delivery of internalized Texas red-EGF from early endosomes into the late endosomes/lysosomes. The DN-LIMK1- (A) or unphosphorylatable S3A cofilin-transfected cells (B) were treated as described for Figure 4, and superimposed images of Texas red-EGF and transferrin receptor (TfR) or Texas red-EGF and LIMPII are shown as indicated. Bar, 10μm.

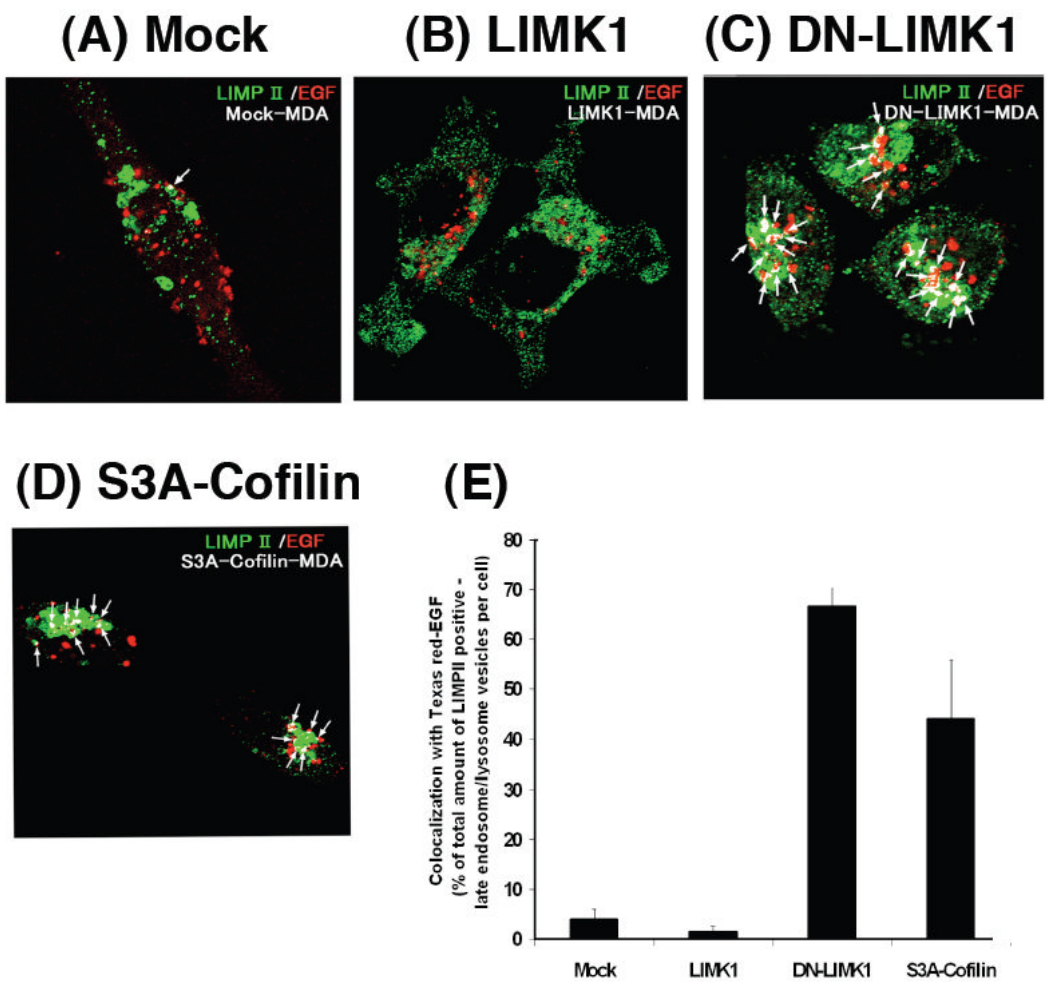

Figure 6 is the syntheses of data published previously by Nishimura et al. in 2006.

Figure 6. Evidence that expression of kinase-deficient dominant negative LIMK1 mutant or unphosphorylatable mutant of cofilin rescues efficient endocytic delivery of EGF-EGFR complex out of early endosomes to the late endosomes/lysosomes in MDA-MB-231 cells. Superimposed confocal images of the internalized EGF and LIMPII-positive structures at 30 min in Mock- (A), LIMK1- (B), DN-LIMK1- (C), or S3A cofilin transfectants (D) are shown. The immunofluorescence intensity profiles of the green and red channels were analyzed in a 2D-histogram as described in Materials and methods, and an area of significant overlap is shown in white pseudo-color imaging of colocalization in the merged micrographs. In (E), the merged images as white color were quantitated and presented as the percentage of total amounts of LIMPII-positive vesicles per cell. The error bar denotes SD.

We further examined the ligand-induced endocytosis of EGFR in the S3A cofilin-transfected cells. The result shows that efficient endocytosis of Texas red-EGF was also seen in the cell after 15-30 min internalization, and that the endocytosed Texas red-EGF-positive small vesicles were colocalized well with the LIMPII-positive late endosomes/lysosomes in the perinuclear region (Figure 5B). These results observed in the S3A cofilin-transfectants are corresponded well with those in the DN-LIMK1 transfectants. Therefore, our data indicate that endogenous kinase activity of LIMK1 has some regulatory role on the endocytic traffic of EGF-EGFR complex out of early endosomes toward late endosomes. Furthermore,

quantitative analysis was carried out to determine the amounts of LIMPII-positive late endosomal/lysosomal marker that colocalized with the endocytosed Texas red-EGF in each transfectants (Figure 6). In the mock or wtLIMK1 transfectants, most Texas red-EGF-positive vesicular structures were distinct from the LIMPII-positive vesicular structures in the perinuclear region of the transfectants (Figure 6A, B). In contrast, in the DN-LIMK1- or S3A cofilin-transfected cells (Figure 6C, D), an extensive overlap was seen between the endocytosed Texas red-EGF staining and the LIMPII-positive late endosomes/lysosomes (quantitated in Figure 6E). Collectively, it is evident that EGF-EGFR traffic out of early endosomes to late endosomes/lysosomes is impaired by LIMK1 expression.

DISCUSSION

In this study, we analyzed the role of LIMK1 on the regulation of EGFR trafficking through the endocytic pathway in human breast cancer MDA-MB-231 cells, which is known to be highly invasive, and the expression levels of LIMK1 is several times higher than that of MCF-7 cells, low invasive cancer cells (Yoshioka et al., 2003). Here, we found a notable suppressive effect on the endocytosis of EGFR, and large amount of EGF staining was accumulated in the early endosomes in the LIMK1-transfected MDA-MB-231 cells. In order to understand the molecular basis of reduced internalization of EGFR in the LIMK1-transfected cells, we further analyzed an effect of kinase-deficient dominant negative LIMK1 mutant on the EGFR-mediated endocytosis. Here, we found an enhanced delivery of Texas red-EGF from early endosomes to late endosomes/lysosomes after 15-30 min internalization in DN-LIMK1-transfected cells, and internalized EGF was degraded in the LIMPII-positive late endosomes/lysosomes after 60 min internalization (data not shown), thereby, indicating that the transfection of kinase-deficient mutant rescues an efficient endocytic trafficking of EGF-EGFR. Moreover, we confirmed these results by employing S3A cofilin mutant-transfected cells. In the S3A cofilin transfectants, we demonstrated reduced levels of phosphorylated-cofilin by immunoblotting analysis, thus, suggesting that LIMK1 activity is considerably suppressed in this transfectant. In these transfectants, vesicular trafficking of Texas red-EGF indeed returned to normal regarding Texas-red-EGF endocytosis, and large amount of EGF staining was colocalized with LIMPII-positive punctate structures in the cell after 15-30 min internalization. These results also demonstrated that LIMK1-cofilin signaling pathway has pivotal role on the efficient translocation of EGFR out of early endosomes toward late endosomes through regulating actin cytoskeleton. In our previous report, we studied the effect of LIMK1 overexpression on the internalization of EGF in MCF-7 cells, and we demonstrated a significant retardation in the internalization of Texas red labeled-EGF and significant accumulation of internalized EGF staining associated with early endosomes instead of being delivered to late endosomes/lysosomes in the LIMK1-transfected cells in comparison with mock transfectants (Nishimura et al., 2004). Collectively, it is reasonable to imply that LIMK1 has potentially suppressive effect on the vesicular traffic out of early endosomes toward late endosomes through regulating cellular actin dynamics.

In receptor-mediated endocytosis, ligand-receptor complexes are internalized and transported via clathrin-coated vesicles to the early endosomes. EGF is a secreted peptide that stimulates cell growth and cell division by binding to a receptor tyrosine kinase at the plasma

membranes, and then EGFR recruits and phosphorylates signaling molecules, leading to activation for such as a MAPK-signal transduction cascade that is important for the regulation of cell growth (Schlessinger, 2004). EGF-EGFR complexes are degraded upon delivery to the lysosomes through endocytosis, which is known as a receptor downregulation. Impairment in the receptor downregulation prolongs cell growth factor signaling, thereby, receptor downregulation is a critical regulatory step in ensuring the correct cell signaling. Therefore, we postulate that LIMK1 is one of the key components on sorting of EGFR from early endosomes to late endosomes /lysosomes where degradation of that complex takes place.

It has been reported that actin cytoskeletal filaments as well as microtubules provide a network important for the movement and positioning of both early and late endosomes as well as lysosomes (Cordonnier et al., 2001). Membrane traffic to and through late endosomes is not only sensitive to microtubule and actin filament depolymerization but is controlled by many proteins that interact with actin and microtubules such as myosins, kinesins, and the dynein/dynactin complex (Ellis and Mellor, 2000; Murphy et al., 1996). Hence, we propose that LIMK1 plays important roles in coordination with microtubule rearrangements through regulating actin cytoskeletons in the fusion events between early endosomes and late endosomes through endocytic pathway. Further study to define the mechanism by which LIMK1 participates in the regulation of sorting of EGF-EGFR out of early endosomes in the endocytic pathway will be required.

Taken together, our data show that LIMK1-cofilin signaling pathway indeed plays an important role in the regulation of ligand-induced translocation of EGFR through the endocytic pathway in invasive human breast cancer cells. Further study to clarify the detailed mechanism how LIMK1-cofilin signaling pathway regulates vesicle traffic of EGFR through the endocytic pathway in human cancer cells is now under investigation.

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Chapter 7

SIALIC ACIDS AS MODULATORS OF ENDOCYTOSIS: THE CASE OF DENDRITIC CELLS

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ABSTRACT

Sialic acids are a family of sugars, which typically terminate cell surface glycoconjugates. Due to this ubiquitous position, they represent key structural determinants for a number of receptors, and are potential modulators of immune responses, among which endocytosis.

Dendritic cells (DC) are professional antigen-presenting cells, crucial for linking innate and immune responses, and have been exploited as therapeutic tools to trigger immunity against tumors or pathogens, or dampen hypersensitivity responses. DCs show a wide capacity to acquire exogenous antigens through different mechanisms, which dictates its immunoregulatory capacity. This chapter focus on the complex role of their highly sialylated cell surface content as modulator of DC endocytosis. In particular it will be presented data that support the evidence that sialylation shortage, while affecting DC maturation, results in opposing effects on macropinocytosis and phagocytosis mechanisms mediated by DCs.

Removal of the cell surface sialylated structures by sialidase treatment decreases the capacity of human monocyte-derived DC (mo-DC) to endocytose antigens, mainly uptaken by macropinocytosis, but not bacteria uptaken by phagocytosis, such as *Escherichia coli*. Sialidase treatment was found to trigger mo-DC maturation and therefore affect the cell actin cytoskeleton organization, explaining somehow macropinocytosis downregulation. Surprisingly, this treatment significantly improves the bacterial uptake by cytokine matured mo-DCs. Mouse models data suggested that the

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sialylated glycans mediated by ST3Gal.I and ST6Gal.I sialyltransferases are related with the observed maturation induction and subsequent decreased macropinocytosis capacity.

The complex role of sialic acid as endocytosis modulator was further enforced by findings revealing that mo-DCs express surface ectosialyltransferases, which rapidly sialylate cell surface and may interfere with mo-DC endocytosis capacity.

Of interest, the findings here described reveal a complex system involving sialylated structures that subverts part of the well known downregulation of bacteria endocytosis typical of mature mo-DC and may have a potential therapeutic application.

INTRODUCTION

Sialic acids or N-acetylneuraminic acids (Neu5Ac) are a family of nine carbon sugars derived from neuraminic acid, typically found on the outermost ends of cell surface glycoconjugates. In humans, sialylation of glycoconjugates is mediated by twenty different sialyltransferase enzymes, using CMP-Neu5Ac, an activated sugar donor, as substrate to catalyze the transfer of sialic acid residues to terminal non-reducing positions of oligosaccharide chains of glycoproteins and glycolipids. Sialyltransferases are usually integral membrane proteins of the Golgi apparatus and generate sialic acid content and diversity during the biosynthesis of glycoconjugates [1]. Depending on their nature, sialyltransferases may establish different types of linkages and are currently divided into four families. The ST3Gal family comprises six members and catalyzes the transfer of Neu5Ac residues in α2,3-linkage to galactose (Gal), while the ST6Gal family has only two known members and catalyzes α2,6-linkages to Gal. There are six known members in each of the ST6GalNac and ST8Sia families, which are able to establish α2,6-linkage to the N-acetylgalactosamine (GalNAc) or α2,8-linkages to another sialic acid, respectively (reviewed in [2]).

1. SIALIC ACIDS AND ITS ROLE IN THE IMMUNE RESPONSE

Due to its terminal position, sialic acids are potential key structural determinants for a number of cell surface receptors involved in the immune response, such as the Siglecs (sialic acid-binding immunoglobulin-like lectins) and selectins (reviewed in [3, 4]). Siglecs are members of the Ig superfamily characterized by their specificity for sialic acids attached to the terminal regions of cell-surface glycoconjugates, which have the potential to interact not only *in trans* with sialylated ligands found in other cells, but also *in cis* with ligands found at same cell surface [5]. Siglecs are differentially expressed on various subsets of leucocytes, but the physiological functions of the majority of Siglecs are still poorly characterized. Nevertheless, they contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which allocate them, to the role in the positive and negative regulation of immune and inflammatory responses, as discussed in recent reviews [3, 6].

Selectins are crucial for leukocyte migration to sites of inflammation and secondary lymphoid organs, since they mediate the capture of free flowing leukocytes to the vessel wall and leukocyte rolling. These glycoproteins bind to carbohydrate determinants on selectin ligands, which are usually sialyl Lewis X antigens [4].

Sialic acid moieties also prevent the deposition of complement on cell surface and are also ligands for a diverse array of exogenous carbohydrate-binding proteins (lectins), from other host cells and invasive pathogens [7].

As an integral part of the systemic inflammatory response, the ST6Gal.I sialyltransferase expression is upregulated and there is a consequent augment of α2,6-linked sialic acid in serum proteins. The physiological role of ST6Gal.I mediated sialylation in inflammation is poorly understood but, among other possible effects, it might create a cell surface shield against pathogens [8]. Mouse gene knockout (KO) studies have also provided an irrefutable evidence of the immune-relevant function of the α2,3 and α2,6-sialylation [9-11]. ST6Gal.I KO mice are reported as having an impaired humoral immune response, evidenced by reduced levels of circulating and surface IgM, impaired B lymphocyte proliferation in response to various activation signals and impaired antibody production in response to antigens [10]. On the other hand, ST3Gal.I KO mice have an almost total absence of peripheral CD8β T lymphocytes, which are lost by apoptosis [9].

There are several other examples of important roles played by sialic acids not only in immune responses but also in other physiological and pathological processes - which include the microbe binding leading to infections and the progression and spread of human malignancies - and in certain aspects of human evolution.

2. DENDRITIC CELLS

Dendritic cells (DCs) are special antigen-presenting cells that uptake, process and present antigens -found in peripheral blood and tissues- to lymphocytes and also regulate cellular and humoral immune responses [12]. Due to their role in initiating the specific immune response, monocyte derived DCs (mo-DCs) have been exploited as therapeutic tools to trigger immune responses against cancer cells or pathogens, or dampen hypersensitivity responses [13, 14].

The uptake and processing of antigens found in the peripheral blood and tissues is performed in a unique way. Immature DCs uptake antigens by several systems: i) receptor mediated endocytosis, which consists in the uptake of small molecules after their recognition by surface membrane receptors, ii) Phagocytosis or “cell eating”, the uptake of large particles, such as apoptotic and necrotic cells, viruses and bacteria, also involving specific cell-surface receptors, iii) macropinocytosis, an actin-dependent process involving membrane ruffling and the formation of membrane vesicles, which is a constitutive process in DCs, allowing the ingestion of large volumes of fluids and solutes [15]. In general, after antigen uptake, the endossomes fuse with the lysossomes within the cell. A subsequent signal cascade prompts the transport of vesicles to the cell surface and the endocytosed antigens are presented at surface through MHC class II (MHC II) or CD1 molecules, or in case of cross presentation, through MHC class I (MHC I).

Besides antigen uptake, inflammatory stimuli also induce DC maturation, enabling DC interaction with T lymphocytes and the initiation of adaptive immune responses [16]. DC maturation is characterized by phenotypic and physiologic changes, i.e. the antigen-uptake machinery is downregulated [17] and the endocytosed antigens are presented, through MHC and CD1 molecules, to resting lymphocytes in secondary lymphoid organs to where antigen-loaded DCs migrate during their maturation process. The expression of costimulatory

molecules, such as CD80 and CD86 [18] and the synthesis of DC specific cytokines [19] is also crucial to determine the type of immune response induced by DCs.

In general, all the factors and stimulus received by DCs, namely, the type of recognized and endocytosed antigens and the extracelular cytokines, seem to be fundamental to skew DCs towards a Th1- or Th2- promoting effector function [20] thus leading to cell-mediated or humoral immunity, respectively.

3. SIALYLATION PROFILE OF DENDRITIC CELLS

Just recently, researchers have started to investigate the pattern of glycosylation and, more specifically, the sialic acid content on human mononuclear phagocyte system (mainly on DCs, macrophages and monocytes). Important and distinct modifications of the pattern of glycosylation in DCs and macrophages undergoing differentiation from monocytes were found, with potential biological consequences [21].

Regarding sialylation, a more perceptive study has been initiated solely in mo-DCs. In particular, binding experiments with plant lectins have given a broad perspective that immature mo-DC surface show a highly α2,6- and α2,3-sialylated phenotype, when comparing with its precursors, the monocytes [22, 23]. *Sambucus nigra* lectin, which recognizes mainly sialic acid α2,6-linked to lactosamine (6' sialyl Galβ1,4GlcNAc), and *Maackia amurensis* lectin, which recognizes mainly sialic acid α2,3-linked to lactosamine (3' sialyl Galβ1,4GlcNAc) or, to a lesser extent, linked to Galβ1,3GalNAc bind strongly to mo-DC. On the other hand, the overall reactivity of *Arachis hypogaea* lectin, a lectin specific for nonsialylated Galβ1,3GalNAc (core 1 structure of the O-linked chains), to mo-DCs is very low, supporting the idea that these cells express the Core 1 structure of the O-linked chains (T antigen) masked by sialic acid.

The analysis of the sialyltransferases mRNA through microarray analysis and quantitative real-time PCR have enlightened the molecular basis of the mo-DCs sialylation profile [21-23]. Mo-DCs sialylation profile is a commitment of several sialyltransferases encoded by different genes. Due to their acknowledged expression and role in other leukocytes [9, 10], a particular relevance has been given to α2,3- and α2,6-sialyltransferases, or, in other words, to ST3Gal, ST6Gal and ST6GalNac sialyltransferases, and not so much to ST8Sias. Among all, the expression of ST3Gal.I and ST6Gal.I has been pointed out by different researchers, since it increases significantly during mo-DC differentiation. This increase has been confirmed at protein level by determining, during mo-DC differentiation, the overall sialyltransferase activity towards precise ST3Gal.I and ST6Gal.I substrates. In fact, a straight correlation between ST3Gal.I and ST6Gal.I gene expression, respective activities and mo-DC α2,3- and α2,6-sialylation phenotype, has been found during monocytes differentiation into DCs [22]. These sialyltransferases are thus considered to be the major responsible for the addition of α2,3- and α2,6- sialic acids or the biosynthesis of α2,3- and α2,6- sialic acids containing structures specific of mo-DCs and are probably related with cell functionalities. Nevertheless, similarly to other leukocytes, a potential contribution of other sialyltransferases, also expressed in mo-DCs and hypothetically involved in the biosynthesis of specific sialylated structures, should not be excluded, as they may interfere with cell-cell interactions. For instance, ST3Gal.IV and ST3Gal.VI may be involved in sialyl Lewis antigen's biosynthesis

and some members of the ST6GalNac and ST3Gal sialyltransferase family may be involved in the biosynthesis of simple mucin type carbohydrates, such as the Thomsen Friedenreich antigens [24].

4. DC ENDOCYTOSIS MODULATION BY SIALYLATION CONTENT

The sialic acid's role on endocytosis has long been studied on an antigen or pathogen perspective. It is now evident that several pathogenic bacteria have evolved to use sialic acid beneficially by coating themselves with sialic acid, providing resistance to components of the host's immune response, or by using sialic acid as a nutrient [25]. Regarding the phagocytic cell, a special attention has been given to the sialylation of dendritic cells and this issue will be discussed below in this chapter.

4.1. The Sialidase Effect in Mo-DCs

Terminal sialyl residues may be removed from glycoconjugates by sialidases (neuraminidases), which comprises a family of glycoside hydrolase enzymes that are widely distributed throughout nature. Some bacterial and viral sialidases have been cloned and characterized and exhibit substrate specificity regarding sialic acid linkage or the presence of substituents. Sialidases, therefore, are an excellent tool for investigation and are frequently employed for *in vitro* studies to allow the desialylation of glycoconjugates from cell surface.

4.1.1. Consequences on Endocytosis Capacity

The effect of sialidase treatment in DCs endocytosis mechanisms has been investigated, comparing the capacity of human mo-DCs treated with sialidase or not, for their uptake of fluorescent or fluorescently labelled antigens, such as ovalbumin, Lucifer yellow, dextran, and killed *Escherichia coli*, in non-opsonic conditions. Except for the final endocytic tracer, which is normally phagocytosed, the others are known to be mainly internalized through macropinocytosis.

Flow cytometry analysis and confocal microscopy visualizations have shown that sialic acid removal clearly influences the endocytic ability of mo-DCs. Indeed, in desialylated mo-DCs, the internalization of ovalbumin, Lucifer yellow and dextran is significantly reduced [22] (Figure 1). Explicitly, according to the endocytic capacity values of the cells, the uptake of ovalbumin is decreased to 67.2% ($p = 0{,}007$), Lucifer Yellow to 71.1% ($p = 0{,}031$) and dextran is decreased to 85.9% ($p = 0{,}007$) when mo-DCs were treated with sialidase, comparing with parallel, identical non-treated mo-DCs (Figure 1) [22].

In contrast the capacity of mo-DCs to uptake *E. coli* is not negatively affected by sialidase treatment even suggesting that desialylated DCs have improved phagocytosis capacity (Figure 1). *E. coli* uptake was confirmed to be mainly performed through phagocytosis and not by macropinocytosis, as evidenced by assays conducted with Rottlerin, a specific inhibitor of macropinocytosis [26] (data not shown). Therefore, since DCs retain a high phagocytic capacity when submitted to sialidase treatment, but less ability for

macropinocytosis, it is probable that removing sialic acid affects specific endocytic mechanisms in distinct ways.

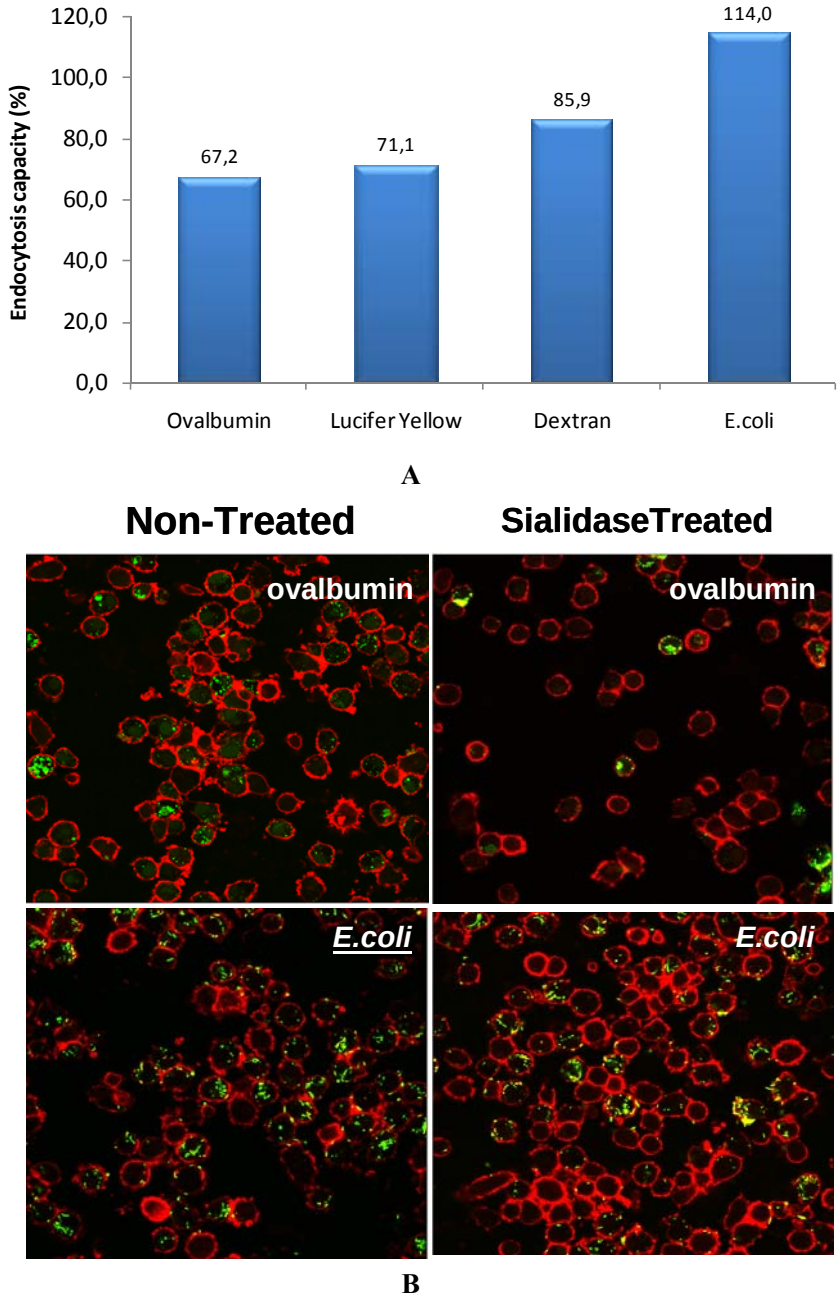

Figure 1. Sialidase treatment affects endocytic capacity of mo-DCs. **A**: Mean percentage of endocytic capacity of mo-DCs treated with sialidase as compared to non-treated mo-DCs (original values were obtained by flow cytometry analysis, as referred in [22]). **B:** Confocal microscopy images confirming that sialidase treated cells have lower capacity to uptake ovalbumin but not *E. coli.* Actin filaments are stained in red and the endocytic agents are in green.

4.1.2. Consequences on Cell Maturation

Another interesting observation is the induction of mo-DC maturation after the exclusion of sialic acid from cell surface by sialidase treatment. This is confirmed by the increased expression of MHC class I and II and costimulatory molecules (CD80, CD86) observed in these cells after treatment. Besides, sialidase treated mo-DCs show a higher capacity to activate and burst T lymphocyte proliferation and trigger the transcription of cytokine genes involved in pro-inflammatory responses [27,16].

Literature usually refers that, in the presence of several factors such as inflammation or "danger" signals, immature DCs undergo a complex process of morphologic and functional activation, leading these cells to rapidly progress from intermediate to fully mature/activated DCs. Concordantly, one can suggest that sialidase treatment has identical effect as inflammation or "danger" signals.

When activated, mature DCs become potent antigen-presenting cells, with augmented cell surface patterning of molecules involved in antigen presentation (MHC class I and II and CD1 molecules) throughout extensive veiled membrane protrusions and increased expression of costimulatory molecules, essential for the activation of T lymphocytes. Along with these extensive phenotypic and morphologic changes, a signal cascade triggers the endocytosis inhibition and, as a result, mature DCs lose much of their macropinocytic and phagocytic capacity [16].

Thus, the more mature phenotype observed in the mo-DCs submitted to sialidase treatment may explain, in part, the less capacity of these cells to uptake certain antigens. However, removal of cell surface sialic acid seems to exert a more complex effect than triggering the maturation of immature mo-DCs, since DCs matured by means of sialidase treatment retain a full or even higher phagocytic capacity (Figure 1).

The influence of sialidase treatment in mature DCs antigen-uptaking capacity was also addressed carrying out endocytosis assays with DCs activated with acknowledged maturation factors such as bacterial lipopolysaccharide (LPS) and TNF alpha. These factors do decrease macropinocytosis and phagocytosis ability of fully sialylated mo-DCs (< 50% in the case of LPS). Although, surprisingly, sialidase treatment of LPS- or TNF alpha-mature mo-DCs significantly improves their capacity to uptake *E. coli* (> 200% in the case of LPS-matured, $p = 0{,}009$), as compared to mature non sialidase treated, whereas decreasing the mature mo-DC ovalbumin uptaking capacity (Figure 2).

In the literature there are a few reports demonstrating that some factors, while able to trigger DC maturation – as confirmed through the phenotypical, morphological and functional cell characterization – still do not significantly interfere with cells' ability to phagocytose antigens [28, 28-31]. As an example, Nayak et al (2006) have already demonstrated that mature DC have reduced capacity to internalize soluble antigens through the formation of solute-bearing vesicles – a characteristic of macropinocytosis – but retain a relative ability to internalize particles, thus suggesting discrete regulation of macropinocytosis and phagocytosis in DCs [28].

Nevertheless, opposing any other maturation triggering factor referred in the literature, the sialidase treatment of the mo-DCs seem to have an unexpected positive effect in phagocytosis capacity. These observations strongly enforce the complexity of effects triggered upon sialic acid removal by sialidase treatment.

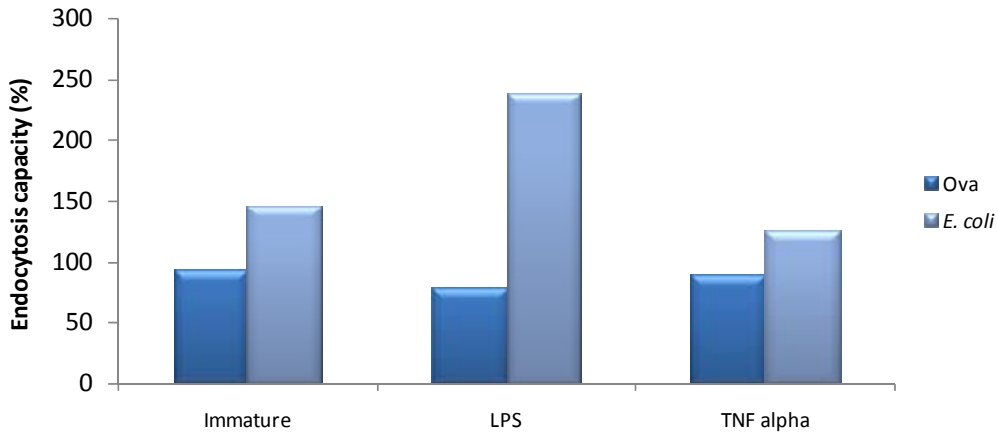

Figure 2. Opposite effect of sialidase treatment in the capacity of immature and LPS- or TNF alpha-mature mo-DCs to uptake ovalbumin (Ova) and *E. coli.* Results represent the mean percentage of endocytic capacity of mo-DCs treated with sialidase as compared to their corresponding non-treated mo-DCs. Original values were obtained by flow cytometry analysis.

4.1.3. Consequences on Macropinocytosis versus Phagocytosis

It is well known that the formation of solute-bearing vesicles (macropinossomes) is largely dependent upon regulation of actin polymerization which in turn is mediated by a number of actin-binding and regulatory proteins, such as Rho family GTPases, namely Rac1 and Cdc42 [32-34]. These Rho GTPases function as molecular switches, cycling between an active and inactive GDP-bound state. Garret et al (2000) found that endogenous levels of Cdc42-GTP is markedly reduced in mature versus immature DCs, suggesting that DCs appear to control the actin-dependent event of endocytosis, at least in part by controlling levels of activated Cdc42 [32-34].

Internalization of bacterial particles is best understood in macrophages where generally two types of mechanisms mediated by opsonins are well characterized. One mechanism involves fragments of complement cascade (C3), which opsonize bacteria and allow their recognition by phagocytes through CR3 receptors. The other mechanism involves antimicrobial antibodies, which react with the appropriate antigenic determinants on the bacteria surface through their Fab combining sites and binds through their FC portion to the corresponding receptor (FcR) on the surface of phagocytes. Both receptor-mediated phagocytosis mechanisms are finely regulated by distinct Rho subfamily GTPases such as Rho, Rac1, and Cdc42 [35].

However, distinct types of recognitions of non-opsonized targets by phagocytes have been described, whose mechanisms surely diverge from either CR3 or FcR pathways. A great part of this non opsonic phagocytosis seems to be accomplished by the interaction of lectins present on the surface of either phagocyte or pathogen that combine with complementary sugars expressed on the surface of the opposite cell, in a lock-and-key manner, leading to phagocytosis [36]. Nevertheless, the molecular bases and mechanism of regulation involved in the non-opsonic phagocytosis are rather veiled.

Regarding the effect of cell surface sialic acid removal, it is obvious that the uptake of the antigens mainly internalized through macropinocytosis in mo-DCs is decreased after sialidase treatment. Since this treatment leads to a mo-DC mature phenotype, intracellular signaling pathways are likely to be activated. This may include the alteration of the signaling pathway of Rho proteins, involved in the cytoskeleton reorganization, a requirement for the macropinocytosis process. Immunofluorescence microscopy studies revealed that it is, in fact, possible that sialidase action influences the signaling that triggers the organization of actin rich ruffles in particles and fluid uptake (Figure 3). However, in sialidase-treated mo-DCs, ovalbumin internalization is not drastically reduced, since the cell may compensate the inhibition of macropinocytosis with an alternative way of capture, such as the receptor mediated endocytosis.

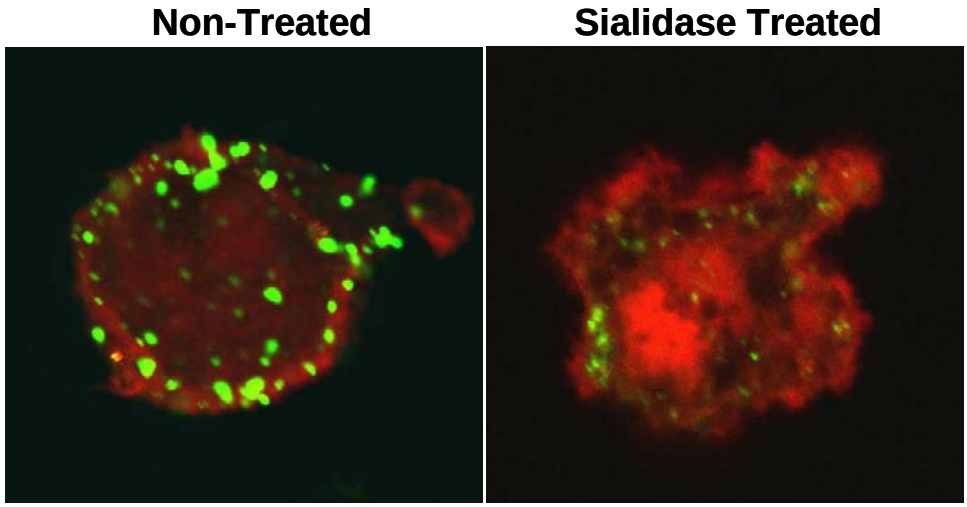

Figure 3. Confocal microscopy images of mo-DCs comparing the feature of actin filaments (red) of sialidase treated cells and non-treated cells, during the endocytosis of ovalbumin (green).

On the other hand, since the assays to analyze the sialidase effect on mo-DCs on the phagocytosis capacity were conducted in non-opsonic conditions, it is expectable that *E. coli* were internalized through non opsonic mechanisms, such as the referred sugar mediated phagocytosis.

It is possible that, within the complexity of the cells surface molecules, sialic acid removal from the cell surface glycoconjugates alters the cell surface repulsive forces that may exist, interfering with adhesion and recognition of bacteria by mo-DC. Besides, there are many possible candidate glycan recognizing receptors for *E. coli* [i.e. mannose receptor, scavenger receptors, Toll-like Receptors (TLR), Siglecs] that may be “unmasked” after sialic acid removal, becoming more suited to recognize glycosidic structures expressed by the bacteria and promoting the adherence and, consequently, phagocytosis. So, even if mo-DCs became more mature after sialidase treatment, it seems that in the case of *E. coli* phagocytosis, in the used experimental conditions, the removal of sialic acid somehow helps this process, especially in DCs pre-matured by other stimulus. Apparently, a combination of different effects, including adhesion and receptor binding capacities, is trigerred by sialidase.

Although sialidase treatment damagingly compromises the actin-dependent antigen uptake mechanisms, it is postulated that phagocytosis, contrarily to macropinocytosis, is

positively influenced by sialidase-mediated desialylation of DCs' glycoconjugates. Clearly, further studies will be necessary to enlighten this potential modulation effect of sialic acid on endocytosis.

4.2. Sialyltransferase Deficient DC

As previously mentioned, during mo-DC differentiation it was observed an increased expression of certain sialyltransferases, namely, ST3Gal.I and ST6Gal.I [22], further strengthening the importance of these sialyltransferases in the immunobiology of DCs. Interestingly, KO mice deficient for ST3Gal.I or ST6Gal.I present impaired adaptive immune response, whose elucidation is not fully accomplished but largely resides on the sialylation of structures specific of T and B lymphocytes such as CD8 and CD22, respectively [9, 10]. Nevertheless, given the role of DCs in orchestrating lymphocyte function, abnormalities at this level are also expected. The question, therefore, is to what extent does ST3Gal.I and ST6Gal.I specific sialylation influence the normal DC processes, namely on endocytosis.

The weight of specific sialic acid linkages in the endocytic process has been evaluated on bone marrow derived dendritic cells (BMDCs) obtained from ST3Gal.I$^{-/-}$ and ST6Gal.I$^{-/-}$ mice. These mice were generated by Prof. Jamey Marth team, for the Consortium of Functional Glycomics, an international research initiative funded by NIGMS created to help the study of the carbohydrate-protein interactions at cell level. Results point to a significant influence of the ST3Gal.I and ST6Gal.I mediated sialylation on endocytosis, with a reduction on the endocytic function of, approximately, 40% when ST3Gal.I-mediated sialylation is suppressed and approximately 20% when no ST6Gal.I-mediated sialylation is present (Figure 4). Also observed was the expression of DC maturation markers, namely, I-A^{b} (mouse MHC class II molecule), is naturally increased in these KO BMDCs. Interestingly, after endocytosis, the normally increased I-A^{b} expression is significantly higher when compared with WT BMDCs, especially in the case of ST3Gal.I$^{-/-}$. The relationship between endocytosis inhibition and the initiation of DC maturation is well known and has already been discussed in the previous section. Thus, the presence of a higher level of maturation markers induced by a reduction of specific sialic acid content could account for the reduction of endocytosis level. Questions such as "Why does the α2,3-sialylation absence influences a higher degree of endocytosis inhibition and maturation, when compared with α2,6-sialylation absence?" or, "What is the mechanism involving specific sialic acid linkages that can influence the enhanced maturation of DCs?" are points that must be addressed in the future and will further enlighten the involvement of sialic acid in the endocytic process.

4.3. Effect of Rapid Sialylation by Membrane Ectosialyltransferases

Even though sialyltransferases are usually integral membrane proteins of the Golgi apparatus, other forms of sialyltransferases have been described, such as soluble or cell surface sialyltransferases [37-40]. In mo-DC there is no evidence for the release of soluble sialyltransferases. However experiments with fluorescent CMP-Neu5Ac substrate which does not penetrate the cell and is highly resistant to sialidases [41] have proven the existence of cell surface sialyltransferase activity which rapidly modulates or restores mo-DC surface sialylation content (Figure 5) [42]. Sialylation mediated by ectosialyltransferases does not

interfere with mo-DC maturation, but it downregulates to nearly one third the ability of mo-DCs to endocytose ovalbumin.

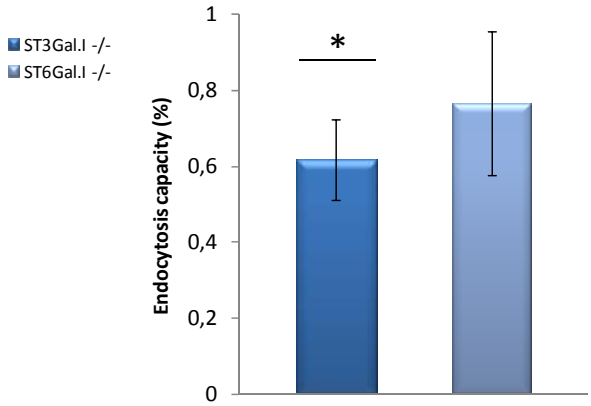

Figure 4. Ovalbumin endocytosis in BMDCs from ST3Gal.I$^{-/-}$ and ST6Gal.I$^{-/-}$ mice. The chart represents the endocytosis capacity of KO-BMDCs compared to the WT-BMDCs' capacity. Original values were obtained by flow cytometry analysis, as referred in [27].

It has been reported by some authors that the sialyltransferase donor substrate, CMP-Neu5Ac is, under certain circumstances, present at extracellular milieu and is probably released by epithelial cells and phagocytes during inflammation [43]. In this scenario, it was hypothesized that, in physiological conditions, the ectosialyltransferases may participate in the complex inflammatory response, modulating the immune system. Whereas in neutrophils it has been reported that rapid sialylation mediated by surface sialyltransferases modulates their capacity to adhere to and migrate across the endothelium, in DCs it inhibits antigen-uptake, which may have specific impact in an inflammatory response.

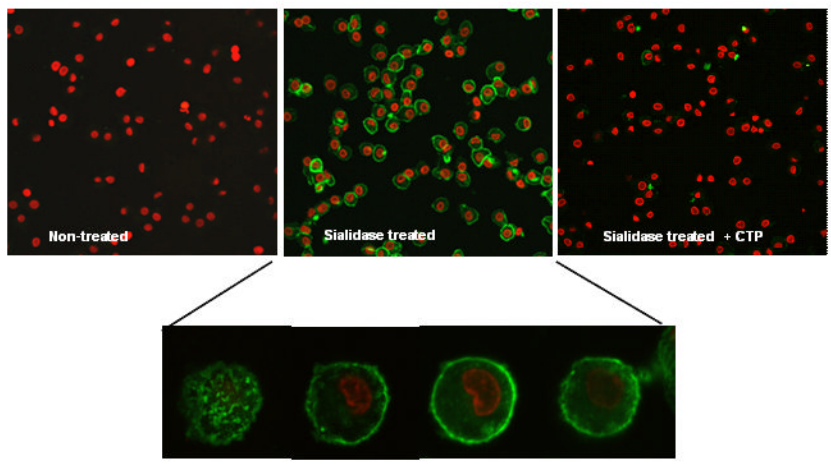

Figure 5. Human mo-DCs incubated with fluorescent CMP-Neu5Ac show evidence of nonintracellular sialyltransferase activity [42]. Confocal laser-scanning microscopy showed that sialidase-treated mo-DCs have a marked incorporation of cell surface sialic acid (green), compared to non-treated mo-DCs and this activity is inhibited by the addition of a sialyltransferase inhibitor, the CTP. Cell nuclei are stained in red.

CONCLUSION

It has been clear that sialylation shortage affects DC maturation functions and results in opposing effects on macropinocytosis and phagocytosis mechanisms mediated by these cells. These findings assume an extreme relevance given the importance of DCs as coordinators of the immune system and its potential application as cellular vaccines to burst an immune response against pathogens or tumoral antigens. At this stage, our understanding of the underlying mechanisms is still limited and the interpretation of the data assumes a considerable difficulty when evoking the diverse receptors that are expressed by DCs. The information regarding the sialic acid content of several receptors known to mediate endocytosis is lacking. While sialylation shortage is presented as a simple manner to modify DC immune functions and to subvert its endocytosis capacity with potential therapeutic applications, an elucidation of these mechanisms at molecule level is still a demand to guarantee its applicability from bench to bedside.

The question of whether the modulation of DC sialic acid content can, undoubtedly, improve DC based vaccines, by dictating the type of antigens that are recognized and the response that is burst by DC remains now open!

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Chapter 8

MACROPINOCYTOSIS: POSSIBLE MECHANISMS OF CELLULAR ENTRY OF ARGININE-RICH INTRACELLULAR DELIVERY PEPTIDES

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ABSTRACT

Endocytosis, which plays a key role in many different species, is the process that cells take up extracellular materials through plasma membranes. Protein transduction domains (PTDs), also called cell-penetrating peptides (CPPs), are small peptides and contain a large amount of basic amino acids. Several PTDs, including arginine-rich intracellular delivery (AID) peptides, were found to be responsible for cellular uptake of macromolecules. In our previous studies, AID peptides have been proven to either covalently transport proteins or noncovalently internalize proteins, DNAs or RNAs into animal or plant cells. The mechanisms by which PTD enter cells are still in vigorous debate. Our studies indicated that the possible mechanisms of AID peptide-mediated cellular entry might involve a combination of multiple internalization pathways, including at least macropinocytosis. Furthermore, our recent reports demonstrated for the first time that AID peptides could rapidly and efficiently deliver proteins into animal and plant cells in both covalent and noncovalent protein transductions (CNPT) synchronously. Therefore, investigations of cellular uptake mediated by AID peptides

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facilitate our understanding of endocytosis in more details and reveal nonclassically endocytic pathways.

INTRODUCTION

Endocytosis is a process that internalizes extracellular material through plasma membranes. This process is obligate for survival as cells apply this tactic to either obtain essential nutrition or to excrete cellular waste. Endocytosis involves formation of vesicles which later fuse with endosomes, and as such, contents from endocytosis usually enter the endo-lysosomal system in the late period of the process [1]. An endocytic pathway involves many basic cellular mechanisms, such as phospholipid recycling of plasma membranes, protein trafficking, cellular polarity maintenance, down-regulating receptors on cell membrane and antigen presentation [1, 2]. Although endocytosis is crucial to cellular survival, it can also be exploited by pathogens and toxins to allow their entry into cells. Thus, voluminous investigations have been launched to study 1) processes and cellular components that are involved in endocytosis and 2) endocytic roles in physiology and pathology. In the late 1990s, endocytosis was classified into clathrin-dependent and clathrin-independent pathways after the discovery of a caveolae-dependent pathway [3, 4]. Most uptake studies on microorganisms and viruses investigated clathrin-dependent and clathrin-independent pathways. To date, studies have proved to be inconclusive in identifying the number of and the kind of cellular components involving clathrin-independent and caveolae-independent endocytic pathways [3]. According to analysis of the ultrastructure of membrane-transport intermediates, endocytic pathways were further classified into four categories: clathrin-dependent, caveolae-dependent, polymorphous tubes formation and macropinocytosis [3].

Tat protein derived from human immunodeficiency virus type 1, discovered in 1988, was thought to have potential in overcoming the barrier of plasma membrane during cellular internalization [5, 6]. Protein transduction domain (PTD), also named as cell-penetrating peptide (CPP), is the functional domain in Tat protein which gives rise to its membrane penetrating ability [7]. Within the domain, it contains a large amount of basic amino acids which are important to its efficiency of protein transduction [8]. Not only can PTD translocate into cells by itself, but it also can transport other cargoes into cells together. These cargoes carried by PTDs vary widely in types, such as proteins, nucleic acids, peptide nucleic acids, nanoparticles and liposomes. The size of these cargoes can be even larger than 200 nm in diameter [9, 10]. In our previous studies, we utilized PTD analogues called arginine-rich intracellular delivery (AID) peptides (i.e., nona-arginine) and demonstrated that AID peptides could deliver biologically active macromolecules into different kinds of cells in both covalent and noncovalent manners [11–17]. The kinetics of cellular entry of PTD peptides were measured at a half-time of 1.8 min, corresponding to a first-order rate constant of 0.007 sec^{-1} at a peptide concentration of 100 μM [18].

The mechanism of PTDs internalization is still in vigorous debate. A previous study indicated that internalization of PTDs is energy-independent [8]; therefore, low temperature could not prohibit cellular entry of PTDs. Kawamura *et al*. demonstrated that p53-Tat fusion protein enters CHO cells via a clathrin-dependent endocytosis [19]. Other investigations supported the notion that PTDs cross-linked with cargoes utilize a caveolae-dependent pathway for internalization in various cells [20–22]. In addition to caveolin-mediated

pathway, recent studies have focused on another major but nonclassical pathway, macropinocytosis, in cellular internalization of PTDs alone or conjugated with cargoes covalently [23–26]. Our group is a pioneer in the study of plant species on transduction mechanisms of AID peptides covalently conjugated with fluorescent protein (FP). Our data indicated that internalization of FP-AID peptides is neither clathrin-dependent nor caveolae-dependent [11]. While we changed the FP and AID peptides linkage from covalent to noncovalent manner, their entry into cells was both receptor- and energy-independent. Further investigation indicated that macropinocytosis was the mechanism of action in delivering AID peptides in various types of cells [13, 15, 17]. Our most recent work demonstrated that AID peptides could enter cells in both covalent and noncovalent protein transductions (CNPT), and that AID peptides could deliver two kinds of FP into cells in covalent and noncovalent fashions synchronously [27].

In this report, we summarize uptake efficiency and mechanistic studies on covalent, noncovalent and combined transduction of various types of cargoes in plant species, mammalian species and cyanobacterial strains. We start this article by describing protocols and procedures of transduction. Particularly, detailed attention focuses on construction and preparation of plasmids from our laboratory, optimization, induction and purification of recombinant proteins, as well as treatment of cells and use of endocytic modulators aimed at various mechanisms of transduction. Data are compared and contrasted between covalent protein transduction (CPT), noncovalent protein transduction (NPT), and CNPT. At the end, we summarize others' and our discovery and point out research for the future.

MATERIALS AND METHODS

Plasmid Construction and Protein Preparation

The pR9 plasmid we constructed contains a hexa-histidine (6His) and a nona-arginine (R9) sequence under the control of the T7 promoter, while the pR9-GFP plasmid we made has an additional coding region of green fluorescent protein (GFP) inserted into the pR9 vector as previously described [11]. The pQE8-GFP plasmid (kindly provided by Dr. Michael B. Elowitz, Rockefeller University, NY, USA) consists of a coding sequence of GFP under the control of the T5 promoter [11]. The mCherry plasmid (kindly provided by Dr. Roger Y. Tsien, University of California, San Diego, CA, USA) has a coding sequence of 6His-tagged red fluorescent protein under the control of the T7 promoter [12]. The pHBT-sGFP(S65T)-NOS plasmid (GenBank Accession No. EF090408, kindly provided by Dr. Jen Sheen, Harvard University, MA, USA) contains an engineered *gfp* gene under the control of the 35S cauliflower mosaic virus enhancer fused to the basal promoter of the maize C4PPDK gene [15]. Plasmid DNA was purified with the Nucleobond AX100 Kit (Machery-Nagel, Duren, Germany). All constructions were verified by DNA sequencing.

For protein expression, pR9, pR9-GFP, pQE8-GFP and mCherry plasmids were transformed into *Escherichia coli* and induced as previously described [13, 27]. Expressed proteins were then purified, concentrated and quantified by the Protein Assay Kit (Bio-Rad, Hercules, CA, USA). The chemically synthetic nona-arginine (SR9) peptide (MDBio, Taipei, Taiwan) with more than 95% purity was described previously [13].

Preparation of Various Cells

Human A549 lung cancer cells (American Type Culture Collection, Manassas, VA, USA; CCL-185) were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (BioWest, Nuaille, France) supplemented with 10% heat inactivated (56°C for 30 min) bovine serum (Gibco, Invitrogen, Carlsbad, CA, USA). Cells were cultured in a humidified 5% CO_2 and 95% air incubator at 37°C as previously described [14, 27].

Onion (*Allium cepa* L.) bulbs, root tips of mung bean (*Vigna radiata* L.), soybean (*Glycine max* L.) and corn (*Zea mays* L.) were prepared as previously described [11, 13, 15]. For cyanobacteria (blue-green algae) culture, both *Synechocystis sp*. PCC 6803 (ATCC, 27184) and *Synechococcus elongatus* PCC 7942 (ATCC, 33912) (kindly provided by Dr. Yuh-Jang Shieh, National Defense Medical Center, Taiwan) were grown in BG-11 medium with mild shaking at 50 rpm and regular illumination at 28°C [17].

Protein Transduction and Treatment of Endocytic Modulators

In the study on CPT, plant cells were mixed with 2.7 μM of R9-GFP covalent fusion protein for different periods of time and washed with distilled water to remove free protein. In the investigation of NPT, R9 or SR9 peptide was mixed with GFP at a molecular ratio of 3:1 at room temperature for 10 min. All kinds of cells and tissues treated with AID peptides/FP noncovalent mixtures were described previously [11, 13, 14, 17]. SR9 peptide was mixed with plasmid DNA at the nitrogen/phosphate (N/P) ratio of 3 for 30 min, then transferred to another eppendorf tube and incubated with plant tissues. After incubation for 30 min, SR9/DNA mixtures were removed by washing with double deionized water [15]. Plant tissues were placed on slides after 48 h of treatment and observed under the microscope. For CNPT test, A549 cells were treated with 10 μM of mCherry premixed with 30 μM of R9-GFP at the molecular ratio (R9-GFP:mCherry) of 3:1 for 10 min at 37°C and washed with phosphate buffered saline [27].

A suite of endocytic modulators and physical procedures were used to study mechanisms of internalization. For energy-dependent experiments at 4°C, the protocol of protein transduction was the same as above except that all incubations were performed at 4°C. Cells were preincubated at 4°C for 30 min before being incubated with the protein transduction solution. For endocytic modulator assays, cells were treated with either R9-GFP, AID peptides/FP mixtures, AID peptides/DNA mixtures or R9-GFP/FP mixtures in the absence or presence of 1 mM of *N*-ethylmaleimide (NEM; Sigma-Aldrich, St. Louis, MO, USA), 1.5 μM of okadaic acid (Sigma-Aldrich), 2 μM of valinomycin (Sigma-Aldrich), 2 μM of nigericin (Fluka Chemie, Seelze, Germany), 10 mM of sodium azide (Fluka Chemie) or 80 mM of sodium chlorate ($NaClO_3$; Sigma-Aldrich), respectively. For macropinocytosis and cytoskeleton motions, cells were treated in the absence or presence of 100 μM of 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA; Sigma-Aldrich), 10 μM of cytochalasin D (CytD; Sigma-Aldrich) or 10 μM of nocodazole (Sigma-Aldrich), respectively. To deplete or sequester cholesterol from plasma membrane and impair lipid raft formation, 2 mM of methyl-β-cyclodextrin (MβCD; Sigma-Aldrich) were added in the culture [11, 13–15, 17, 27].

Confocal Microscopy

Images were observed under an inverted TMS microscope (Nikon, Melville, NY, USA) equipped with a MD130 CMOS sensor (Electronic Eyepiece, Dar-An, Taiwan) or under an Eclipse E600 microscope (Nikon) and recorded by a Penguin 150CL cooled CCD camera (Pixera, Los Gatos, CA, USA). Fluorescent images were acquired by the TCS SL confocal microscope system (Leica, Wetzlar, Germany), and relative intensities of fluorescent images were quantified by the UN-SCAN-IT software (Silk Scientific, Orem, UT, USA) as previously described [17].

Statistical Analysis

Results were expressed as means ± standard deviations (SDs). Statistical comparisons between the control and treated groups were performed by Student's *t*-test. The levels of statistical significance were set at $P < 0.05$ (*).

RESULTS

Mechanisms of Covalent Protein Transduction (CPT) for Protein Delivery

In order to reveal mechanisms of cellular internalization of AID peptides in a covalent manner, plant cells were treated with R9-GFP fusion protein at different time points in the presence or absence of various inhibitors. R9-GFP was internalized within 5 min in cells (Figure 1, control), while other controls were described previously [11]. The fluorescence emitted by internalized protein could remain in plant cells stably more than 2 days after protein transduction. Low temperature (4°C) incubation and treatment of endocytic inhibitors (NEM and okadaic acid) did not alter AID peptide internalization in CPT manner (Figure 1). These results suggested that cellular internalization of R9-GFP was independent of energy and classical endocytosis.

Mechanisms of Noncovalent Protein Transduction (NPT) for Protein Delivery

For cellular internalization of AID peptides in a noncovalent fashion, cells from different species were treated with AID peptides/GFP mixtures. In the absence of drugs or physical treatment, protein internalization in plant cells was detected at 5 min of incubation with R9/GFP mixtures (Figure 2A, control). Similar to the results in CPT in plants, low temperature and endocytic modulators, such as NEM, okadaic acid, valinomycin, nigericin and sodium azide did not interfere AID peptide internalization in NPT (Figure 2A). These results suggested that either energy-dependent or classical endocytosis pathway was not the major pathway in NPT. Contrarily, treatment of plant cells with macropinocytosis inhibitors EIPA and CytD resulted in a dramatic reduction in cellular entry indicating that NPT in plant cells involve macropinocytosis and actin rearrangement.

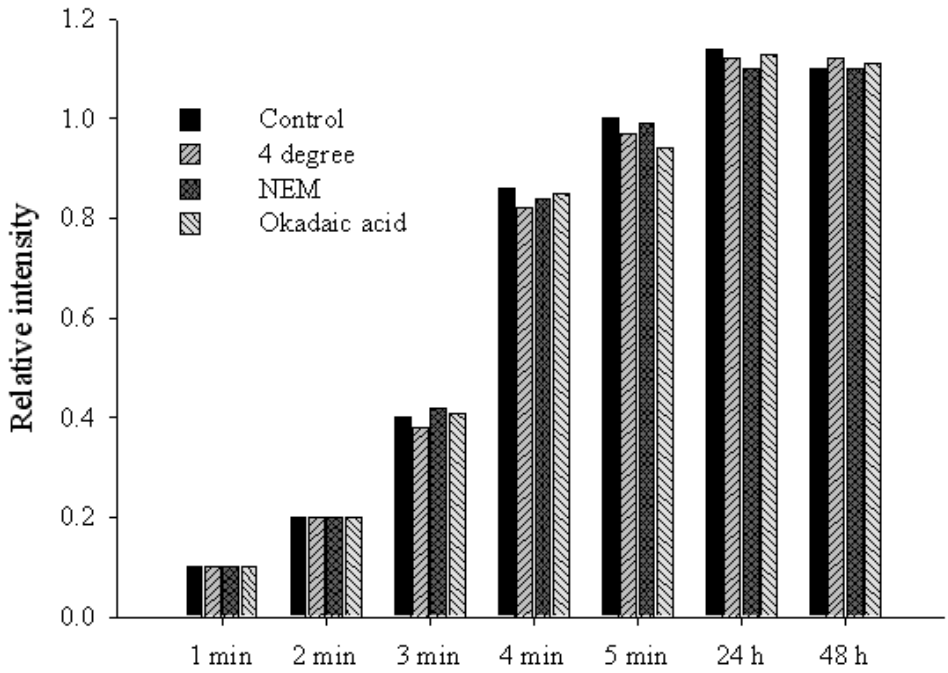

Figure 1. Mechanisms of covalent protein transduction (CPT) for protein delivery. Onion epidermal cells were treated with R9-GFP in the absence (control) or presence of 1 mM of NEM or 1.5 μM of okadaic acid at room temperatures. Cells were also treated with the same protein solution at 4°C. Relative fluorescent intensities (y-axis) at different times (x-axis; 1, 2, 3, 4, 5 min, 24 h and 48 h) after protein internalization were analyzed with the UN-SCAN-IT software.

Similar results of NPT were observed in mammalian cells (Figure 2B). Human A549 lung cancer cells treated with AID peptides/GFP mixtures for 20 min at 37°C in the presence of EIPA or CytD dramatically decreased NPT.

Interestingly, cyanobacteria displayed the ability of GFP uptake without facilitation by AID peptides (Figure 2C). Therefore, in order to test the mechanism of NPT in AID peptides/GFP mixtures, we chose various endocytic modulators to inhibit the GFP uptake in cyanobacteria. Both energy depletion and classical endocytic modulators successfully prevented the GFP uptake with NEM having the highest prohibitive capacity (Figure 2C). Cells were treated with NEM and R9/GFP mixtures in the absence or presence of EIPA or CytD. Both PCC 6803 and 7942 strains did not internalize R9/GFP in the presence of EIPA. Uptake inhibition by CytD was only observed in PCC 7942 strain. These data suggested that AID peptide-dependent NPT in cyanobacteria was mediated by macropinocytosis.

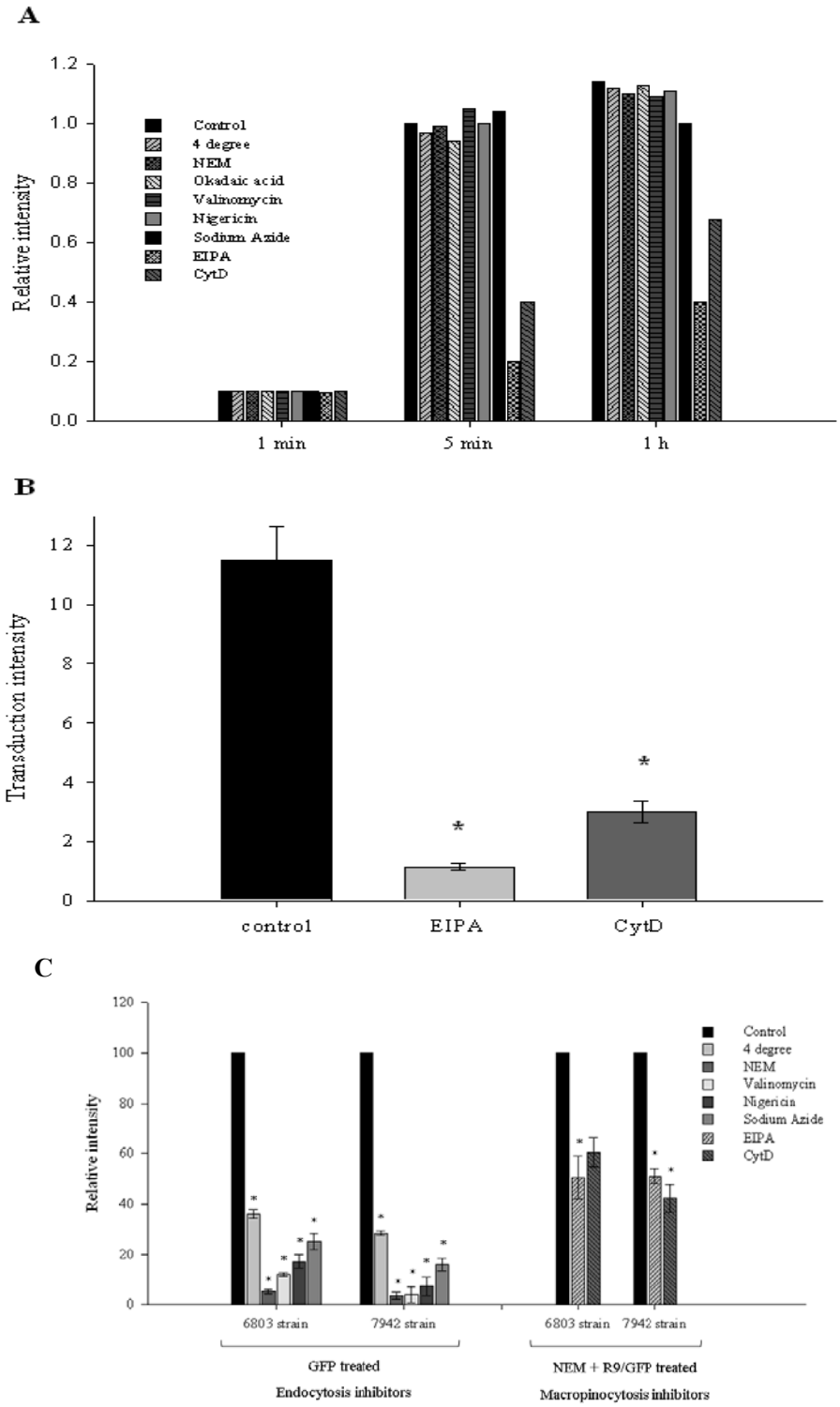
A
Relative intensity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
4 degree
NEM
Okadaic acid
Valinomycin
Nigericin
Sodium Azide
EIPA
CytD
1 min
5 min
1 h
B
Transduction intensity
12
10
8
6
4
2
0
control
EIPA
CytD
C
Relative intensity
120
100
80
60
40
20
0
Control
4 degree
NEM
Valinomycin
Nigericin
Sodium Azide
EIPA
CytD
6803 strain
7942 strain
6803 strain
7942 strain
GFP treated
Endocytosis inhibitors
NEM + R9/GFP treated
Macropinocytosis inhibitors

Figure 2. Mechanisms of noncovalent protein transduction (NPT) for protein delivery in different species. (A). The mechanism of NPT in plant cells. Onion epidermal cells were treated with AID peptides/GFP mixtures in the absence (control) or presence of 1 mM of NEM, 1.5 μM of okadaic acid, 2 μM of valinomycin, 2 μM of nigericin, 10 mM of sodium azide, 100 μM of EIPA or 10 μM of CytD at different temperatures (room temperature or 4°C). Relative intensities at different time (1 min, 5 min and 1 h) were analyzed with the UN-SCAN-IT software. (B). The mechanism of NPT in mammalian cells. A549 cells were treated with SR9/GFP mixtures for 20 min at 37°C in the absence (control) or presence of EIPA or CytD at the same concentrations as described above. Relative intensities in different treatment were represented by mean ± SD. Significant differences were presented as an asterisk (*, p □ 0.05). (C). The mechanism of NPT in cyanobacteria. Two cyanobacterial strains, PCC 6803 and 7942, were incubated with GFP only in the absence (control in GFP treated group) or presence of endocytic modulators (1 mM of NEM, 2 μM of valinomycin, 2 μM of nigericin, or 10 mM of sodium azide) in either room temperature or 4°C. Cyanobacteria were treated with NEM and AID peptides/GFP mixtures in the absence (control in NEM + SR9/GFP mixtures) or presence of 100 μM of EIPA or 10 μM of CytD at room temperature. An asterisk indicates significant differences (p □ 0.05) between the control and the experimental group.

Mechanisms of Noncovalent Protein Transduction (NPT) for DNA Delivery

To understand the mechanism of AID peptide-dependent DNA entry into plant cells in NPT, plant cells were treated with SR9/pHBT-sGFP(S65T)-NOS plasmid mixtures for 30 min in the absence or presence of different kinds of endocytic modulators. After 48 h of incubation in the absence of modulators, the *gfp* reporter gene was expressed and the emission of green fluorescence represented the degree of plasmid DNA translocation via AID peptides in cells (Figure 3, control). Surprisingly, fluorescent intensities in the treatment groups with EIPA, CytD, low temperature, sodium azide or NEM were significantly reduced, while fluorescent intensities in the treatment of valinomycin or nigericin were similar to that of the control (Figure 3). These results indicated that the mechanism of AID peptide-mediated DNA internalization in NPT might differ from those of CPT and NPT for protein delivery.

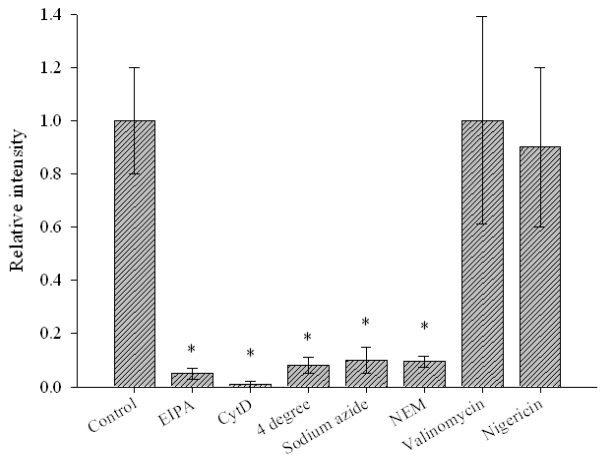

Figure 3. Mechanisms of noncovalent protein transduction (NPT) for DNA delivery. SR9 peptide was mixed with the pHBT-sGFP(S65T)-NOS plasmid at the N/P ratio of 3. Root tips of mung bean were treated with the peptide/plasmid mixtures in the absence (control) or presence of 100 μM of EIPA, 10 μM of CytD, 10 mM of sodium azide, 1 mM of NEM, 2 μM of valinomycin or 2 μM of nigericin for 30

min at room temperature. Samples were also treated with the same mixtures at 4°C. Relative intensities of fluorescence were quantified after 48 h of treatment. An asterisk indicates significant differences (p □ 0.05).

Mechanisms of Covalent and Noncovalent Protein Transductions (CNPT) for Protein Delivery

To elucidate mechanisms of CNPT for protein delivery, human A549 cells were treated with R9-GFP/mCherry mixtures. We found that green fluorescent intensities in all treatment groups with endocytic modulators were significantly reduced except for the 4°C treatment compared to that of the control (Figure 4, R9-GFP group). Contrarily, red fluorescent intensities decreased in treatment with EIPA, CytD or nocodazole, but remained similar intensities in treatment with low temperature, MβCD or sodium chlorate (Figure 4, mCherry group). Moreover, the highest transduction reduction was the group treated with CytD, an inhibitor of actin rearrangement, which involved many kinds of physiological process (Figure 4). These results demonstrated that mechanisms of CNPT differed from those of CPT and NPT for protein delivery. Potential uptake mechanism of CNPT may involve a combination of multiple internalization pathways.

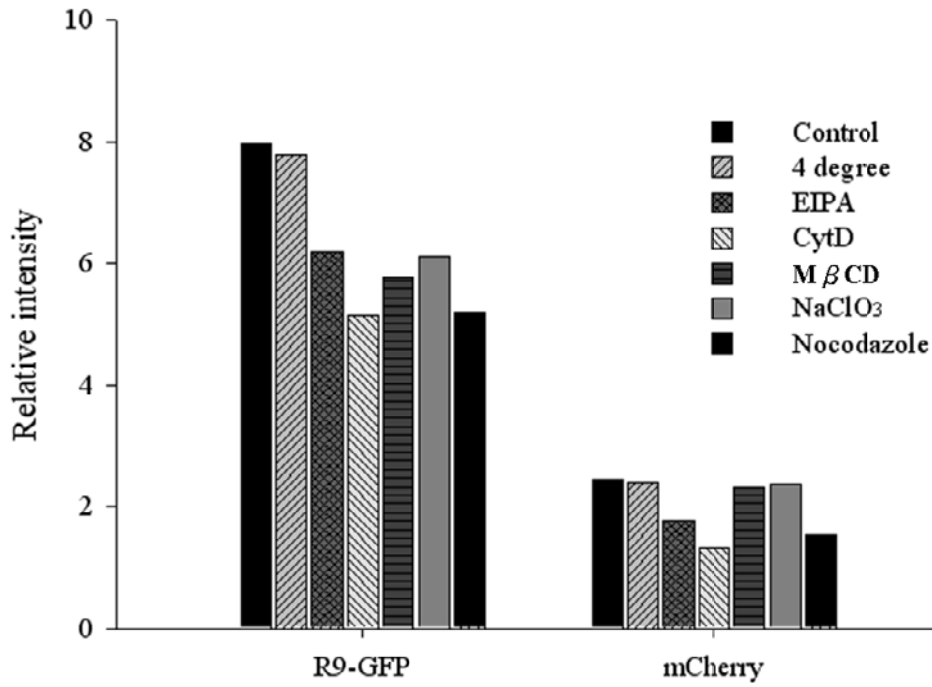

Figure 4. Mechanisms of covalent and noncovalent protein transductions (CNPT) for protein delivery. A549 cells were treated with R9-GFP/mCherry mixtures at the molecular ratio of 3:1 in the absence (control) or presence of different modulators (100 μM of EIPA, 10 μM of CytD, 2 mM of MβCD, 80 mM of sodium chlorate or 10 μM of nocodazole) at different temperatures (37°C or 4°C). Green and red fluorescent images were quantified respectively with the UN-SCAN-IT software and were presented as relative intensities (y-axis).

CONCLUSION

In this study, we have demonstrated that AID peptides possess the ability to efficiently deliver different types of cargoes including proteins and DNAs into various species in covalent, noncovalent or combined covalent and noncovalent manners. Not only can these cargoes be translocated into and stay in cells, but they also retain their activities for a certain period of time. The histograms of CPT and NPT showed that R9-GFP and AID peptides/GFP complexes could internalize into cells as fast as 1 min after treatment, and fluorescent intensities reached near maximum after incubation for 5 min (Figure 1A and 2A). Various types of modulators were chosen to study mechanisms of uptake: classical endocytic modulators (low temperature, NEM, valinomycin, nigericin, sodium azide and sodium chlorate), macropinocytosis inhibitors (EIPA and CytD), cytoskeleton motion inhibitors (CytD and nocodazole) and a lipid raft inhibitor (MβCD). Treatment with classical endocytic inhibitors or energy depletion was unable to prevent AID peptides from internalization by CPT, NPT and CNPT in plant and mammalian cells (Figure 1, 2 and 4). However, EIPA and CytD were effective inhibitors which significantly reduced protein transduction (Figure 2B, 2C, 3 and 4). Collectively our results indicated that macropinocytosis is the major pathway for AID peptides internalization. Yet, it is likely that AID peptide-mediated DNA delivery in NPT and protein transport in CNPT involve a combination of multiple endocytic pathways.

Findings in other reports were consistent with ours in regards to energy-independent CPT, NPT and CNPT. Results from different laboratories indicated that Tat-associated peptides, such as polyarginine or AID peptides, entered cells via an energy-independent manner [7, 8, 10, 25]. Whether internalization of PTDs is mediated by classical endocytosis remained debatable. Recently, endocytic pathways were further divided into ten different types according to their molecular characteristics and implicated cargoes [28]. Endosome membrane potential collapsing drugs (valinomycin and nigericin), a metabolism depression drug (sodium azide) and general endocytic inhibitors (NEM and okadaic acid) were thought to interfere with classical endocytosis [29–31]. However, our data demonstrated that AID peptides were not affected by these drugs in CPT and NPT (Figure 1 and 2). A study showed that PTD entered cells via a clathrin-dependent endocytosis [19], while others proposed caveolae-dependent endocytosis as the major route for PTD internalization [20–22].

Macropinocytosis, first described in 1931, projected distinct structural ruffles on the plasma membrane, and this finding extended the understanding in endocytosis [32, 33]. Macropinocytosis is accompanied by a rapid increase in uptake and extensive leading-edge membrane ruffling that can be observed in 10 min in the experiment [34]. After internalization, inward movement of mature macropinosomes to the perinuclear region involves many cytoskeleton motions, including myosin, dynein and microtubule [35]. Macropinocytosis is sensitive to EIPA and CytD [23, 24]. The EIPA is a Na^+/H^+ exchanged inhibitor which specifically down-regulates ionic equilibrium in macropinocytosis [23, 24, 34]. CytD is an F-actin polymerization inhibitor which disrupts actin dynamics. Our data (Figure 2–4) revealed that AID peptide-mediated internalizations were reduced by treatment with EIPA and CytD. It is worth noting that PTD promoted undefined actin motion and induced the formation of lamellipodia [36, 37]. Together, a lot of endocytic phenomena including classical endocytosis and macropinocytosis were related to actin rearrangement [19, 36].

The size of plasma membrane invaginations may influence routes of internalization. Caveolae-dependent endosomes containing cholesterol, sphingolipids, receptors and caveolins were about 50–80 nm in size [38]. Clathrin-coated pits were around 100–200 nm [39]. Macropinosomes formed from macropinocytosis ranged from 0.2 to 5 μm in diameter [40]. It provided a route for macromolecules to enter cells by a nonselective endocytosis, and large molecules fused or mixed with PTD complexes were likely to be carried out by macropinocytosis. However, size is not the only factor determining the route as molecule charges on the cationic peptides offer strong interactions with heparin sulfate [38]. Therefore, the nature of cargoes plays a determining role in the mechanism of AID peptide-mediated entry as well.

Cell types and condition could also affect on the mechanism of AID peptides uptake [41, 42]. Recent studies suggested that AID peptides entered cells efficiently at 4°C via direct translocation, but cells might uptake AID peptides by macropinocytosis, pinocytosis or direct translocation at the temperature which was higher than 12°C [43]. Factors influencing uptake include, but not limit to, concentrations of AID peptides alone or molecules combined with AID peptides and environmental temperature. According to these investigations, it was noted that there were a lot of factors, which were able to determine and change the mechanism of AID peptide-mediated cellular uptake. Our studies also provided the evidence to support this proposition (Figure 1–4).

Collectively, we have demonstrated that feasibility of AID peptide-mediated cellular uptake of exogenous substances. AID peptides can enter various types of cells of animals, plants and microorganisms by CPT, NPT and CNPT. Specifically, by using modulators and physical treatment we have demonstrated that AID peptides can be internalized via a combination of multiple pathways. More importantly, routes of internalization are species specific [17]. These investigations shed light on details in endocytosis and provided further applications for the future.

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ABBREVIATIONS

AID	arginine-rich intracellular delivery;
CNPT	covalent and noncovalent protein transductions;
CPP	cell-penetrating peptide;
CPT	covalent protein transduction;

CytD	cytochalasin D;
EIPA	5-(*N*-ethyl-*N*-isopropyl)-amiloride;
FP	fluorescent protein;
GFP	green fluorescent protein;
6His	hexa-histidine;
MβCD	methyl-β-cyclodextrin;
NEM	*N*-ethylmaleimide;
N/P	nitrogen/phosphate;
NPT	noncovalent protein transduction;
PTD	protein transduction domain;
R9	nona-arginine;
SR9	synthetic nona-arginine.

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Chapter 9

SYNDAPIN AND SYNAPTIC ENDOCYTOSIS

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ABSTRACT

The molecular mechanisms underlying recycling of synaptic vesicles in nerve terminals are the subject of intense investigation. In this review we consider recent progress in this field with a focus on syndapin 1, a candidate endocytic protein distinguished by its membrane-sculpting and actin remodeling properties. Syndapin 1 interacts with dynamin and the actin-regulator N-WASP, and it contains a F-BAR domain which effectively deforms membranes. In vertebrates, perturbation of syndapin 1, or of its interaction with dynamin does not influence the clathrin-dependent pathway used for synaptic vesicle recycling at moderate rates. However, synaptic vesicle recycling during high levels of synaptic activity is markedly disrupted by these treatments. This distinct role of syndapin probably reflects an involvement in bulk endocytosis, a pathway yet to be defined in molecular terms. Additionally, on the postsynaptic side, syndapin participates in clathrin-mediated uptake of a distinct set of neurotransmitter receptors during development. The synaptic role of syndapin may be limited to vertebrates as genetic deletion of the single syndapin isoform in Drosophila does not affect synaptic function. Syndapin may thus not be regarded as a core component of the clathrin-dependent synaptic vesicle recycling machinery, because it functions in a subset of pre- and postsynaptic endocytic pathways.

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Key words: actin, endocytosis, dynamin, N-WASP, PACSIN, synaptic vesicle, syndapin.

INTRODUCTION

At synapses the fusion of neurotransmitter-filled synaptic vesicles is followed by local recycling of vesicles via endocytosis. Such recycling is critical as failure of this process rapidly leads to depletion of synaptic vesicles with impairment of synaptic transmission along with swelling of the nerve terminal plasma membrane. Rapid progress has been made in characterizing the molecular machinery that mediates synaptic vesicle recycling. In this review, we discuss recent advances in this field with a focus on syndapin 1, a candidate endocytic protein that has recently been the subject of functional studies in different model systems.

The main pathway for synaptic vesicle recycling involves clathrin-mediated endocytosis in the periactive zone (Brodin et al., 2000; Granseth et al., 2006). In brief, this endocytic process begins with recruitment of clathrin, adaptors, and different accessory proteins to a membrane patch (Jung and Haucke, 2007; Dittman and Ryan, 2009). The coated membrane patch grows into a coated pit which successively invaginates until a deeply invaginated pit with a narrow neck has formed. The GTPase dynamin accumulates around the neck and promotes membrane fission (Takei et al., 1995; Pucadyil and Schmid, 2008). The membrane-deformation that occurs during the endocytic process is thought to depend both on the properties of clathrin and on membrane-bending accessory factors, such as the ENTH domain protein epsin and the BAR domain protein endophilin (Itoh and De Camilli, 2006; Ungewickell and Hinrichsen, 2007; Jakobsson et al., 2008; Frost et al., 2009). The latter proteins share the ability to convert large liposomes into narrow tubules. Endocytosis also depends on membrane phosphoinositides which are regulated by the phosphoinositide phosphatase synaptojanin and the kinase PIP1Ɣ (Cremona et al., 1999; Di Paolo et al., 2004; Mani et al., 2007). Other critical accessory factors include the scaffolding proteins Eps15 and intersectin (Marie et al., 2004; Koh et al., 2007). Actin has also been implicated in synaptic vesicle endocytosis, but its role appears to differ between synapse types (Shupliakov et al., 2002; Sankaranarayanan et al., 2003; Richards et al., 2004).

While clathrin-mediated endocytosis is the dominating pathway at low to moderate rates of activity (Granseth et al., 2006; Balaji and Ryan, 2007) an alternate mode, bulk endocytosis, may be used at high rates of activity. This mechanism involves formation of deep membrane infoldings, which may be either pinched off (Wu and Wu, 2007) or remain connected with the plasma membrane (Takei et al., 1996; Gad et al., 1998). Synaptic vesicles are thought to form by clathrin-mediated budding from the infolded membrane as indicated by the presence of coated pits on such structures (Takei et al., 1996; Shupliakov et al., 1997; Ferguson et al., 2007). Key questions regarding this recycling mode still remain open. First, the molecular mechanisms which underlie internalization and vesiculation of membrane are largely undefined. Second, the physiological role of bulk endocytosis is not fully clear. In some cases bulk endocytosis has been shown to occur under physiological stimulation conditions (Teng and Wilkinson, 2000; Paillart et al., 2003; LoGiudice and Matthews, 2007; Cousin, 2009), but many of the studies documenting this phenomenon have relied on non-physiological stimulation (Heuser and Reese, 1973; Takei et al., 1996; Gad et al., 1998; Marxen et al.,

1999; Leenders et al., 2002; de Lange et al., 2003). It is thus likely that bulk endocytosis is used at some, but perhaps not all, synapses.

SYNDAPIN 1

Syndapin 1 was identified in a screen for binding partners to dynamin (Qualmann et al., 1999), in parallel with another study (Plomann et al., 1998). Based on its enrichment in nerve terminals, syndapin 1 was proposed to participate in dynamin-dependent synaptic vesicle endocytosis (Qualmann et al., 1999). Consistent with this possibility, expression of syndapin fragments was shown to inhibit clathrin-mediated endocytosis (Simpson et al., 1999; Modregger et al., 2000; Qualmann and Kelly, 2000; Kessels and Qualmann, 2002). Moreover, syndapin 1 was found to interact with N-WASP, which led Qualmann and colleagues to propose a model in which syndapin 1 promotes endocytic fission by linking dynamin and actin (Qualmann et al., 1999; Qualmann et al., 2000). Interest in syndapin was spurred by the discovery that it belongs to the BAR superfamily of membrane-interacting proteins (Peter et al., 2004). The N-terminal part of syndapin 1 comprises an F-BAR domain (Figure 1) which, like other BAR domains, dimerizes into a curved module with a concave lipid-binding surface (Wang et al., 2009). It is joined via a linker region with a SH3 domain that mediate interactions with dynamin, N-WASP and other proteins (Kessels and Qualmann, 2004). The linker region contains NPF motifs that mediate interactions with eps15 homology domain proteins (EHDs) (Braun et al., 2005).

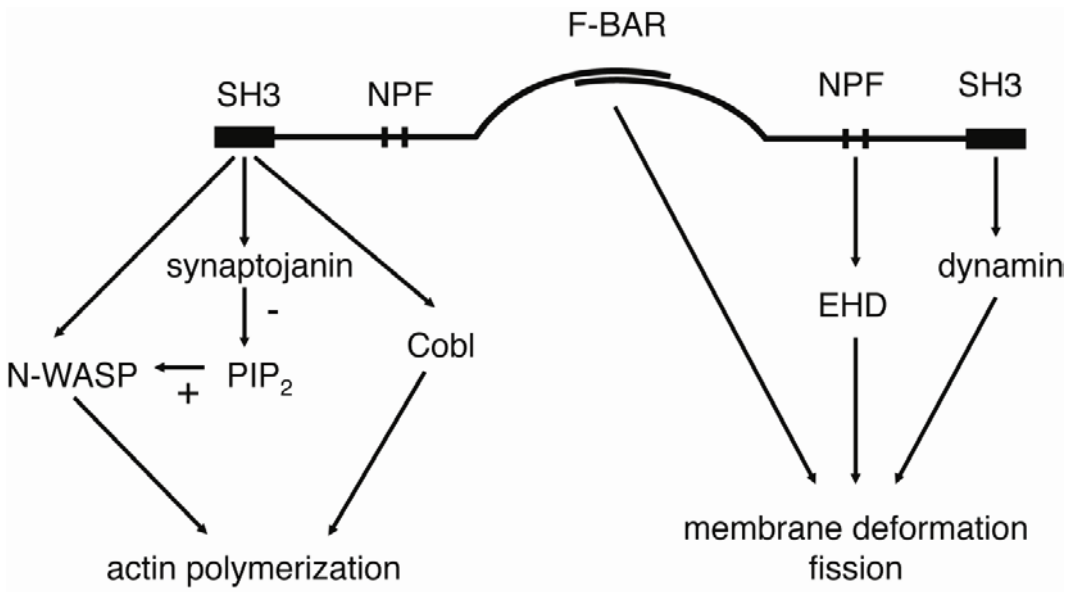


Figure 1. Schematic view of the syndapin F-BAR dimer and its associated network of proteins. The N-terminal part of syndapin consists of a F-BAR domain which after dimerization can directly bend membranes. It interacts with two other membrane-deforming factors, the GTPase dynamin, implicated in fission, and the ATPase EHD. Syndapin can influence the actin cytoskeleton in different ways. It can stimulate formation of branched actin filaments via N-WASP, and straight filaments via Cobl. It can also inhibit actin polymerization via the phosphoinositide phosphatase synaptojanin.

The F-BAR domain of syndapin effectively converts liposomes to tubules. The morphology of these tubules differs to some extent from those induced by other F-BAR proteins tested. While F-BAR domains of proteins such as CIP4 and FBP17 generate large, straight tubules, the syndapin F-BAR domain produces thinner tubules with a variable morphology (Shimada et al., 2007; Frost et al., 2008; Wang et al., 2009). This property has been linked with distinct structural features of the F-BAR dimer (Wang et al., 2009). The liposome-tubulating effect is only observed with the isolated F-BAR domain, suggesting that it is autoinhibited by the linker region and/or the SH3 domain (Itoh et al., 2005; Wang et al., 2009). The role of the F-BAR domain has been recently tested in an *in vivo* context in Drosophila (Kumar et al., 2009b). Transgenic expression of full-length syndapin in muscle was shown to cause a prominent expansion of a tubulolamellar structure, the subsynaptic reticulum, in which N-WASP is enriched. The expansion was abolished after point mutation of residues in the F-BAR domain required for membrane binding. When the F-BAR domain alone was expressed, its targeting to synapses failed, but patches of deformed membrane occurred randomly on the muscle surface (Kumar et al., 2009b). Thus, in this system the syndapin F-BAR domain appears to drive complex membrane remodeling while the SH3 domain is required for targeting, and probably also activation and coordination with actin remodelling.

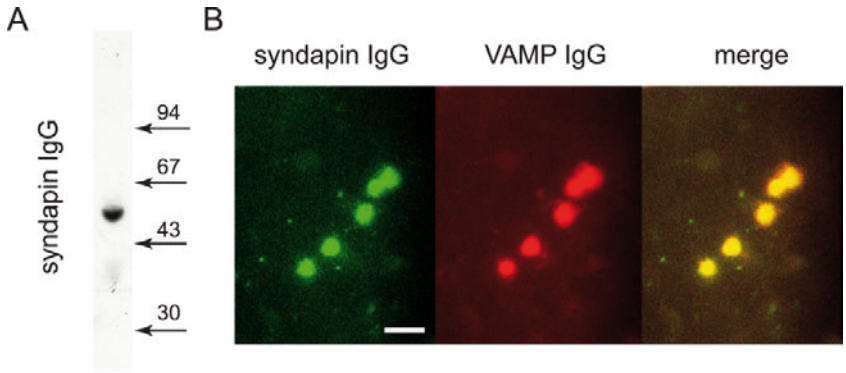
Links between syndapin and the actin cytoskeleton (Figure 1) may be provided not only via N-WASP, but also by interaction with synaptojanin (Kessels and Qualmann, 2004), which dephosphorylates PIP_2 in the plasma membrane (Di Paolo and De Camilli, 2006). As PIP_2 relieves the autoinhibition of N-WASP, synaptojanin can inhibit actin polymerization. In addition, syndapin interacts with Cordon Bleu (Cobl), an actin-nucleating factor that promotes polymerization of straight, unbranched filaments (Ahuja et al., 2007), contrasting with the branched filaments generated by the N-WASP-regulated Arp2/3 complex (Pollard, 2007).

TESTS OF SYNDAPINS ROLE IN SYNAPTIC VESICLE ENDOCYTOSIS

In mammals the syndapin family consists of three members. In addition to the neuron-specific syndapin 1 isoform, it includes the ubiquitously expressed member syndapin 2 and syndapin 3, which is mainly expressed in muscle. In Drosophila a single isoform is present, which is expressed both within and outside the nervous system, including the subsynaptic reticulum (see above). Kumar et al (2009b) recently addressed the involvement of syndapin in synaptic vesicle recycling. The protein was, however, not detectable presynaptically at the larval neuromuscular junction (Kumar et al., 2009a). Moreover, in syndapin-deficient larvae synaptic transmission and synaptic vesicle endocytosis, as measured optically, proceeded normally. Hence, the presynaptic function of syndapin (see below) may be limited to vertebrates.

The role of syndapin 1 in synaptic vesicle endocytosis has been tested in two vertebrate models, the giant reticulospinal synapse in the lamprey (Figure 2) and granule cell cultures from rat cerebellum. Antibody perturbation of syndapin in the reticulospinal synapse did not affect endocytosis at low levels of stimulation; synaptic transmission was unaffected and stimulus-induced clathrin-coated pits appeared in normal numbers in the periactive zone

(Andersson et al., 2008). In granule cell synapses, inhibition of the syndapin-dynamin interaction neither had any effect on endocytic marker uptake at low rates of activity (Clayton et al., 2009).

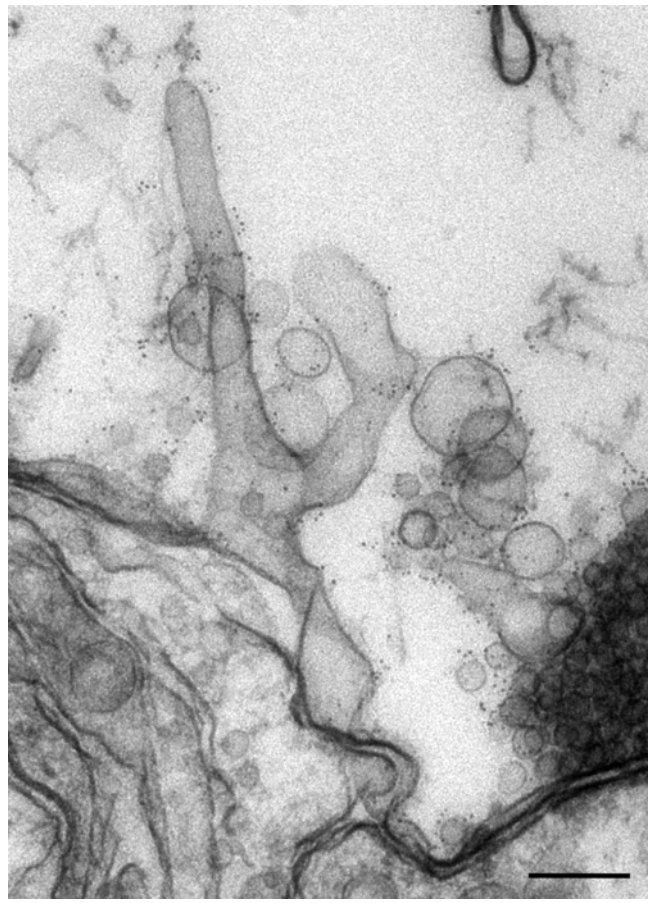


Reproduced from Andersson et al. 2008.

Figure 2. Syndapin is enriched at presynaptic release sites. A shows an immunoblot of lamprey CNS extract demonstrating the selective recognition of a 50 kD band by syndapin antibodies. B. When these antibodies are injected into a giant reticulospinal axon they accumulate at release sites identified by co-microinjected antibodies to the synaptic vesicle protein VAMP. Scale bar=5µm.

In contrast with low frequency stimulation conditions, both Clayton et al. (2009) and Andersson et al. (2008) found marked effects of syndapin perturbation when stimulation in the upper physiological range was used. The study of Clayton et al. (2009) centered on the syndapin – dynamin interaction. The SH3 domain of syndapin binds dynamin at a distinct site in the proline-rich domain which contains two serine residues (774 and 778). These serines are constitutively phosphorylated, which inhibits the interaction with syndapin (Anggono et al., 2006). Clayton et al. (2009) found that in cerebellar neurons dephosphorylation of the serine residues by calcineurin occurs in response to stimulation, but only at stimulus levels that are sufficient to induce bulk endocytosis. In these neurons, bulk endocytosis is seen as uptake of large dextrans and, at the EM level, as occurrence of large cisternae. Inhibition of the syndapin – dynamin interaction with inhibitory peptides inhibited both of these correlates of bulk endocytosis (Clayton et al., 2009). Moreover, knock-down of syndapin by RNAi inhibited dextran uptake.

Andersson et al. (2008) found in the reticulospinal synapse that synaptic responses evoked by high frequency stimulation were depressed by syndapin antibodies and that the synaptic vesicle pool was reduced. Moreover, large cisternae (Figure 3) containing synaptic vesicle membrane, as defined by immunolabelling for VAMP, accumulated in synaptic regions. These cisternae were often seen to remain connected with the plasma membrane. The effect was SH3 domain-dependent and actin labeling in the periactive zone was reduced in a stimulus-dependent manner, consistent with involvement of the syndapin-N-WASP interaction (Andersson et al., 2008).



Reproduced from Andersson et al. 2008.

Figure 3. Perturbation of syndapin followed by action potential stimulation leads to formation of large plasma membrane infoldings. The micrograph shows a 250 nm-thick section of an axon microinjected with syndapin antibodies, stimulated at 5 Hz for 30 min and then maintained at rest for 15 min. The axon was cut open and labeled from the cytoplasmic side with VAMP2 antibodies. Note the small gold particles associated with membrane cisternae and synaptic vesicles. Scale bar=0.2μm.

Both of the above studies are compatible with a role of syndapin in bulk endocytosis. It may be noted, however, that the effects of perturbing syndapin function were not identical in the two systems. In cerebellar neurons cisternae occurred in stimulated control synapses and disappeared after inhibition of the syndapin-dynamin interaction. In the reticulospinal synapse, on the other hand, few cisternae were present in stimulated control axons, but many appeared after perturbation of syndapin. The data obtained in cerebellar synapses point to a role of dynamin-syndapin in the onset of bulk endocytosis, while those in the reticulospinal synapse indicate a role of syndapin in the breakdown and vesiculation of cisternae. It is possible that syndapin acts at both stages, and the differences between the two studies may have been due to the different reagents used. For instance, the inhibitory peptides and the mutagenesis (Clayton et al., 2009) may have spared N-WASP, which was clearly influenced by syndapin antibodies and Fab fragments (Andersson et al., 2008). An additional possible explanation for the effect of syndapin antibodies could be that inhibition of actin polymerization destabilized the plasma membrane in the periactive zone leading to

uncontrolled membrane infolding. In any event, the studies of Andersson et al (2008) and Clayton et al (2009) provide converging evidence for a critical role of syndapin in synaptic vesicle recycling during high-frequency firing. Further analysis of the precise role of syndapin will most likely provide better insight into the mechanisms of bulk endocytosis and its functional importance.

POSTSYNAPTIC ENDOCYTOSIS

Syndapin 1 also occurs postsynaptically where it has been linked with a distinct form of endocytosis (Perez-Otano et al., 2006). In the rodent brain mature glutamatergic synapses contain NMDA receptors with NR1 and NR2 subunits. Another subunit, NR3A, is expressed when synaptic circuitry is established, but is removed as synapses mature (Roberts et al., 2009). The developmental elimination of NR3 receptors has been linked with syndapin 1 (Perez-Otano et al., 2006). Expression of full-length syndapin 1 stimulated, while a dominant-negative construct inhibited endocytosis of NR3A in hippocampal neurons. The inhibitory effect was abolished by point mutations in the NPF motifs and could be mimicked by expressing the NPF region alone. The NPF region was found to interact directly with the cytoplasmic tail of NR3A, and immunoprecipitation experiments demonstrated aggregation of NR3A with syndapin, dynamin and clathrin (Perez-Otano et al., 2006). This study gives the first hint about a functional role of NPF interactions with syndapin. It further points to a role of syndapin in clathrin-mediated receptor endocytosis, although in a very select form. Neither GluR1 receptor endocytosis nor transferrin endocytosis were found to be inhibited by dominant negative constructs of syndapin 1 (Perez-Otano et al., 2006).

CONCLUSION

Understanding the mechanisms of synaptic vesicle recycling is an important goal in synapse biology. In comparison with the extensive analysis of the clathrin-dependent recycling pathway, less attention has been paid to bulk endocytosis. The identification of syndapin as a component which may be selectively used in bulk endocytosis provides new opportunities to study this mechanism. A major question to address is how the proper membrane region (i.e., that which contains vesicle membrane) is recognized and how it is drawn into the cytoplasm. Other interesting questions concern the interplay between the components of the syndapin 1 network—how is the membrane-sculpting effect of the F-BAR domain integrated with actin remodeling, and how are these in turn coordinated with the membrane-tubulating and fission-promoting activities of dynamin and EHD.

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ABBREVIATIONS

Arp 2/3	actin-related protein 2/3
BAR	BIN-amphiphysin-RVS
Cobl	Cordon Bleu
EH	Eps15 homology
EHD	Eps15 homology domain-containing protein
F-BAR	FER-CIP4 homology, BIN-amphiphysin-RVS
N-WASP	Neural Wiskott-Aldrich-syndrome protein
NMDA	N-methyl-D-aspartate
PIP_2	phosphatidylinositol-4,5-bisphosphate
PACSIN 1	PKC and CK2 substrate in neuron 1
PCH	Pombe-Cdc15 homology family of proteins
SH3	src homology 3
syndapin 1	synaptic dynamin-associated protein 1

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Chapter 10

ENDOCYTOSIS: A PROMISING APPROACH FOR GENE TRANSFER TECHNOLOGY

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ABSTRACT

Recently, a number of new methods for gene transfer have been put forward to advance biomedical study and gene therapy. Although viral vectors confer high efficiency of gene transfer, they also have the added risk of carcinogenesis, such as via random genomic integration. Although non-viral vectors lack this risk, they also tended to be less efficient for gene transfer. Thus, various efforts are being made to improve gene transfer techniques based on non-viral vectors. For example, novel chemical transfection reagents have been developed, including those with improved extracellular binding, engulfment, and utilization of the introduced nucleic acids. Additionally, receptor-mediated endocytosis has been used to create vectors using chemicals, growth factor peptides, extracellular matrix proteins, and viral proteins. This method enables selection of target cells and increased gene transfer efficiency as compared with other non-viral methods. In reviewing reports of these efforts, we have found that in terms of efficiency, differences among cells are a bigger factor than differences among transfection reagents. Some cells are always more easily transfected than others. The biological state of a given cell type, such as which endocytic pathways are functional in the cell prior to treatment, may be an important factor in endocytosis-mediated gene transfer. Therefore, studying how endocytosis is regulated should provide useful insights into how to improve gene transfer technology. Endocytosis can be initiated by the binding of ligands to their receptors on the surface of the cell membrane, followed by internalization. To make best use of this endogenous function for introduction of exogenous molecules, it will be necessary to gain a better understanding of what types of

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molecules efficiently induce initiation of endocytosis. In this commentary, we summarize the current state-of-the-art in non-viral gene transfer and supporting technologies related to the initiation of endocytosis. We will also discuss specific ways in which what is known about endocytosis might be exploited in order to facilitate development of new and improved non-viral methods for gene transfer**.**

Endocytosis, a normal cellular activity, can be harnessed as a non-viral method for introduction of nucleic acids into cells. The specific endocytic pathways that are active differ in different cells and thus, cells may respond differently to different transfection reagents aimed at harnessing the endocytic machinery. These cell-type specific differences may account, at least in large part, to the differences in efficiency of the same reagents when used with different cell types. Lipid-based reagents generally provide better performance than polymer-based reagents *in vitro* whereas for endothelial cells, lipid-based methods work poorly or not at all.

In order that plasmid DNA can enter into cells, the exogenous nucleic acids must bind to cationic lipids or polymers to form electrostatic complexes (lipoplexes or polyplexes). Positively charged lipoplexes can then interact with cell-surface anion proteoglycans, termed glycosaminoglycans (GAGs). Adding to the complexity, heparin, heparan sulfate, hyaluronan, chondroitin sulfate, dermatan sulfate, and keratan sulfate can all participate in the interaction. Cells with low levels of GAGs show low efficiency gene transfer by DOTIM [1]. This is attributable at least in part to the fact that free heparin in the transfection medium can competitively inhibit the binding of lipoplexes to cells. On the other hand, overexpression of syndecan-1, a proteoglycan rich in heparan sulfate, resulted in high efficiency transfection. Following their interaction with GAGs, lipoplexes are thought to enter cells via clathrin-mediated endocytosis [2]. Because types and amounts of GAGs vary among cells and tissues, the activity of endocytosis of lipoplex is also likely to vary among cells and tissues.

At first, use of the lipoplex method seemed promising, as lipoplex-mediated transfection produced excellent results *in vitro*. Unfortunately, however, lipoplex-mediated transfection did not work well *in vivo*, which might be attributable to instability of the complex in the presence of serum and aggregation of complexes when present at the high concentrations necessary for *in vivo* transfection [3]. Recently, another cationic lipid emulsion has been developed [4]. These appear to be more stable and effective than traditional cationic lipid emulsions. Further study of the efficiency and safety of these methods for *in vivo* transfection will be necessary to fully evaluate the utility of these alternative lipid-based reagents [5].

A variety of polymers for polyplex have been developed for transfection of plasmid DNA. The application of enzymatic treatments to remove GAGs inhibits transfection with the polymer PLL-polyplex [6]. Moreover, PEI-polyplex internalization is supported by syndecan 1 [7]. As for lipoplexes, it seems likely that GAGs are involved in internalization of these polyplexes, as modulation of GAGs changes the efficiency of gene transfer mediated by at least some of these polyplexes. Many polyplexes similarly rely on a clathrin-dependent pathway for cellular internalization. However, the details of the mechanism of entry of polyplexes vary depending on the types of polymers used and the cells to be treated. Additionally, there is evidence that some types of polyplexes can act via multiple pathways [8].

Internalization has been well studied for at least one of the polymers that is available commercially for *in vitro* and *in vivo* transfection; namely, linear polyethylenimine (lPEI). The pathway(s) involved in internalization of lPEI have been analyzed using inhibitors such as chlorpromazine, which affects the clathrin-dependent pathway for internalization, and with filipin III or genistein, which antagonize the caveolae-dependent pathway. In the hepatocarcinoma cell line HUH-7, the lPEI polyplex is taken up via the clathrin-dependent pathway. However, in HeLa and COS-7 cells, internalization of lPEI polyplex appears to be taken up via caveolae-dependent pathways [9-10]. Given the differences in the responses of different cell-types, selection of the best polymers for a given target cell-type is sometimes critical for effective gene transfer.

As discussed above, lipids and polymers can configure complexes with nucleic acids and undergo endocytosis by themselves. One approach to improving the efficiency of endocytosis of DNA complexes and transfection of cells would be to harness endogenous molecules related to endocytosis as part of the transfection approach. An example of an appropriate starting-point for development of a transfection approach based on the endogenous cellular machinery for internalization is extracellular ligands. Many ligands bind to cell surface receptors and subsequently, the ligand-receptor complexes are endocytosed by the cell. As such, when the complexes are targeted to an appropriate subcellular compartment, they may be effective carriers of DNA complexes.

One example is epidermal growth factor (EGF) receptor, which upon binding of an EGF ligand is endocytosed by the cell, a process that is essential for EGF-mediated signal transduction. Reportedly, DNA complexes conjugated to EGF are taken up by cells via EGF receptors efficiently, even when expression levels of the receptor are low [11-13]. Use of this general approach may improve efficiency. Although increasing efficiency of transfer is a good goal, it is important to balance efficiency with safety. One important factor is whether or not the transfection reagent affects cell proliferation because, as mentioned earlier, transfection comes with a general risk of carcinogenesis. Promisingly, a novel peptide was identified as a ligand for EGF receptor and shown to induce receptor-mediated endocytosis without generating a concurrent increase in cell proliferation, and has a reduced risk of carcinogenesis as compared with other similar approaches [14]. Antibodies that bind to cell-surface receptors are similarly internalized into cells via endocytosis and thus, present another possible route to harnessing an endogenous system for internalization of exogenous DNA molecules [15]. The use of a receptor-specific antibody to generate a complex that is internalized thus presents anther twist on the idea of using ligand-receptor interactions to mediate transfection. The fact that antibodies against the HER2 receptor have successfully been used as a cancer treatment is encouraging, as it suggests that the approach may be safer than alternative approaches.

High levels of expression of folic acid receptor (FR) is specifically associated with some malignant cells and as such, detection of FR has been used to identify malignant cells and then efficiently target them for destruction in both *in vitro* and *in vivo* studies [16-17]. More specifically, FR-α is a useful marker for cancer and FR-β can be used as marker for myeloid leukemia [18]. The artificial modification of ligands that distinguish between these FR subtypes could improve cell and tissue specificity of gene transfer.

Chorea toxin (CTX) is generally used as a marker of caveolin-dependent endocytosis but can also used as a ligand to aids internalization of PEI polyplexes into cells. It has been reported that CTX specifically binds to neural, prostate, and intestinal cancer cells and to

sarcoma cells [19]. Thus, CTX is another promising candidate for a "guiding ligand" useful for introduction of exogenous DNA specifically into the sub-set of cells that are malignant [20].

Recently, another focus of the drug delivery field has been on cell-penetrating peptides. These peptides tend to be rich in arginine and lysine, which should play major roles for their internalization. The peptides under study include endogenously-encoded peptides identified from various organisms, including the HIV-TAT peptide and a peptide present in the *Drosophila* Antennapedia protein. Indeed, TAT peptide modification of lipoplexes has been shown to lead to an increase in transfection efficiency as compared with lipoplex alone [21]. In addition to 'natural' peptides, chemically synthesized poly-arginine peptides (such as repeats of four or up to sixteen arginine residues, R4-R16) have also been studied with considerable intensity. For hematopoietic cell lines, it has been shown that HIV-TAT peptide and R8 can be internalized via the endocytic pathway [22]. Based on the results of chemical inhibitor studies, internalization of TAT and poly-arginine peptides appears to rely on the caveolae-dependent pathway [23]. By contrast, it appears that the Antennapedia peptide may act via a different pathway, at least when present at high concentrations [24-25]. Continued study of peptide-mediated internalization should help to reveal the mechanisms of peptide-based internalization and may help blaze a new path for safe and effective gene transfer.

Arginine-glycine-aspartic acid (RGD) conjugated polymers have also successfully been used accomplish selective transfection to a sub-set of target cells, in this case endothelial cells expressing integrin $\alpha v\beta 3$ or $\alpha v\beta 5$, and are commercially available [26-27]. Integrins present on the cell surface of adherent cells inhibit internalization of cholesterol-enriched microdomains. Detachment of cells from extracellular matrix or integrin binding with soluble ligands, such as the RGD peptide, results in internalization of integrins. Based on this, it seems likely that RGD-conjugated polyplexes might be taken up by cells via this pathway of internalization**.**

Integrins have been found to regulate caveolin-1-mediated internalization of membrane domains that are enriched in lipids such as cholesterol [28]. Thus, use of caveolae-dependent endocytosis for transfection is closely linked to the behavior of integrins. Integrins also form complexes with syndecans, which are proteoglycans with GAGs, and syndecan-1 specifically has been shown to regulate the activity of integrin $\alpha v\beta 5$ and $\alpha v\beta 3$ *in vitro* [29-30]. Integrins are also known to cooperate with several growth factor receptors, including EGFR Excellent reviews have been published on the signal transduction [31-32]. It is possible that integrin binding to RGD peptides might trigger not only internalization of caveolae but also of syndecans and growth factor receptor proteins. Integrin could be a good target for stimulating several routes of internalization [7].

Combinations of DNA complexes and ligands make a molecule with two active domains for extracellular binding. One is a lipid or a polymer, and the other is a ligand for receptor. The precise mechanisms that account for the advantage of two-domain DNA complexes, such as RGD-PEI, for internalization into cells are not well understood. One possibility is that endocytosis starts with the formation of multi-component structures on the surface of cell membranes and that this process is somehow facilitated by the presence of two-domain DNA complexes. Because they are involved in endogenous formation of multi-component structures on the cell surface, integrins are likely to be involved in this process, and as such,

may themselves be an exciting new route to the development of alternative methods of endocytosis-mediated transfection.

Recently, Kitano *et al*. reported that an EGF motif from the extracellular matrix protein Del1 enhances the efficiency of gene transfer *in vitro* [33]. Del1 has three EGF motifs and the RGD sequence present in the second EGF motif has been shown to bind to integrin αvβ3 and αvβ5. The presence of a recombinant, third EGF motif in a Del1 construct increases the efficiency of transfection into a couple of cells types as mediated by either lipoplex or polyplex. Moreover, the effect can be partially inhibited by addition of the inhibitors chlorpromazine or nystatin. It seems possible that the presence of a third EGF in Del1 may activate both endocytic pathways in an integrin-related manner. Given the early promising results with Del1, it seems likely that Del1 may be widely useful to improve the efficiency of transfection.

Despite successful use of a variety of non-viral vectors for transfection, there remain some cell lines and tissues that cannot be easily transfected with the available reagents. One possibility is that these specific cell types lack factors that are critical for specific endocytotic pathways, either at the cell surface or within the cell. Analysis of specific types of cells to determine what factors may be absent, as well as other approaches, may help to overcome the problem. For example, it may be possible to induce expression of specific endocytotic factors in these cells. Moreover, even in cell types that can be transfected, efficiency may be improved as we learn more about endocytosis and how to harness it for internalization of experimentally introduced materials such as DNA. One requirement would be that cells must have the ability to mount an endocytic response to an introduced agent or agents in an organized fashion and rapidly. Towards this goal, it will be important to gain a better understanding of how molecules at the membrane surface are organized and coalesce. More generally, as we gain a better and better understanding of endocytosis, we should be able to develop increasingly sophisticated, safe and effective methods for gene transfer.

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Chapter 11

RAB5 MEDIATED CAVEOLAE ENDOCYTOSIS

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ABSTRACT

Caveolae are morphologically flask-shaped invaginations of the plasma membrane identified in various types of cells such as adipocytes, endothelial cells and myocytes. Unlike clathrin-coated pits, caveolae are detergent insoluble, cholesterol- and sphingolipid-rich, and show high caveolin expression (of caveolin-1, -2 and -3). A large number of molecules have been identified to localize to the caveolae including G-protein coupled receptors, membrane receptors and small GTPases. Recent research has made it clear that caveolae can participate in endocytosis and that they are morphologically distinct from clathrin-coated pits. Caveolin-1, the constitutive protein of the caveolae, is implicated in processes of vesicular transport during caveolae-mediated endocytosis. However, the precise molecular mechanisms of caveolae-mediated endocytosis remain unclear. Rab5 is a small GTPase involved in clathrin-coated vesicle formation, vesicle-early endosome fusion, and early endosome homotypic fusion as well as endosome maturation. Rab5 cycles between the GDP- (inactive) and GTP-bound (active) forms, a process that is tightly controlled by several Rab5 associating proteins. Here we discuss how and when Rab5 activity is controlled during the caveolae-mediated endocytosis and especially focus on the crucial role of the scaffolding domain (SD domain) of cavelolin-1. We also suggest the importance of Rab5 and caveolin-1 interaction during the intracellular trafficking of caveolae on the plasma membrane to the early endosome and to one of their final destination, the Golgi apparatus.

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Keyword: Rab5, caveolae, endocytosis.

BACKGROUND

Caveolae are morphologically flask-shaped invaginations of the plasma membrane identified in various types of cells such as adipocytes, endothelial cells and myocytes (1). Unlike clathrin-coated pits, caveolae are detergent insoluble (2), cholesterol- and sphingolipid-rich, and show high caveolin expression (of caveolin-1, -2 and -3) (3). A large number of molecules have been identified to localize to the caveolae including G-protein coupled receptors, membrane receptors and small GTPases (4, 5).

Interestingly, the caveolae is internalized after stimulation such as the B subunit of cholera toxin (CTXB) and Simian virus (SV40) and is first observed in the early endosome compartment, similar to clathrin-dependent endocytosis. Thereafter, it is transported either to the late endosome or to the Golgi apparatus, depending on the type of stimuli. During caveolae internalization, a clathrin-independent but caveolin-1-containing endosome termed a "caveosome" has been postulated (6,7). This compartment can be separated from the early and recycling endosomes. It is also likely to function constitutively in non-infected cells during transport of sphingolipids and GPI-linked proteins from the plasma membrane to the Golgi apparatus (8,9).

Rab5 is a small GTPase localized on early endosomes, and it controls early endosome fusion during clathrin-dependent endocytosis (10). Emerging data indicate that numerous proteins can selectively bind to Rab5 to facilitate discrete steps in membrane transport (11,12). Pelkmans *et al*. have provided evidence that caveosomes and early endosomes communicate *via* pathways regulated by Rab5 (7). However, the molecular mechanisms of these processes remain unclear. Here, we present some of our recent work as well as our working hypothesis as to how caveolin-1 may control Rab5 activation and intracellular trafficking.

IDENTIFICATION OF CAVEOLIN-1 AS A BINDING PROTEIN OF RAB5

Rab5 activity controls the tethering of transport vesicles to target membranes (13). During this process, the activity is highly regulated by so called "effectors", which in turn regulate the vesicular tethering/fusion events (Figure 1). Most of them either participitae in inactivation (GAP: GTPase-activating protein, GDI: GDP dissociation inhibitor) or activation (GEF: Guanine nucleotide exchange factor) of the Rab5. Membrane recruitment of effectors by these GTPases is therefore crucial to the regulation of vesicular transport events. We recently reported a convenient and cost-effective method to identify novel Rab5-binding proteins and found over 15 candidates (14). On the other hand, analysis of rat spleen homogenate using immuno-precipitation and a magnetic bead based pull-down method allowed us to enrich a caveolin-1 positive organelle from spleen, and Rab5 was present in this fraction. Additionaly, far-Western blotting analysis using bacterially expressed GST-fused dominant active (Rab5Q79L) and inactive Rab5 (Rab5S34N) with His tagged caveolin-1 (His-caveolin-1) indicated that caveolin-1 can directly bind to Rab5, especially in its activated

form (15). Therefore, it is clear that activated Rab5 binds to caveolin-1, and this interaction is predicted to be important for intracellular trafficking of caveolae to the early endosome.

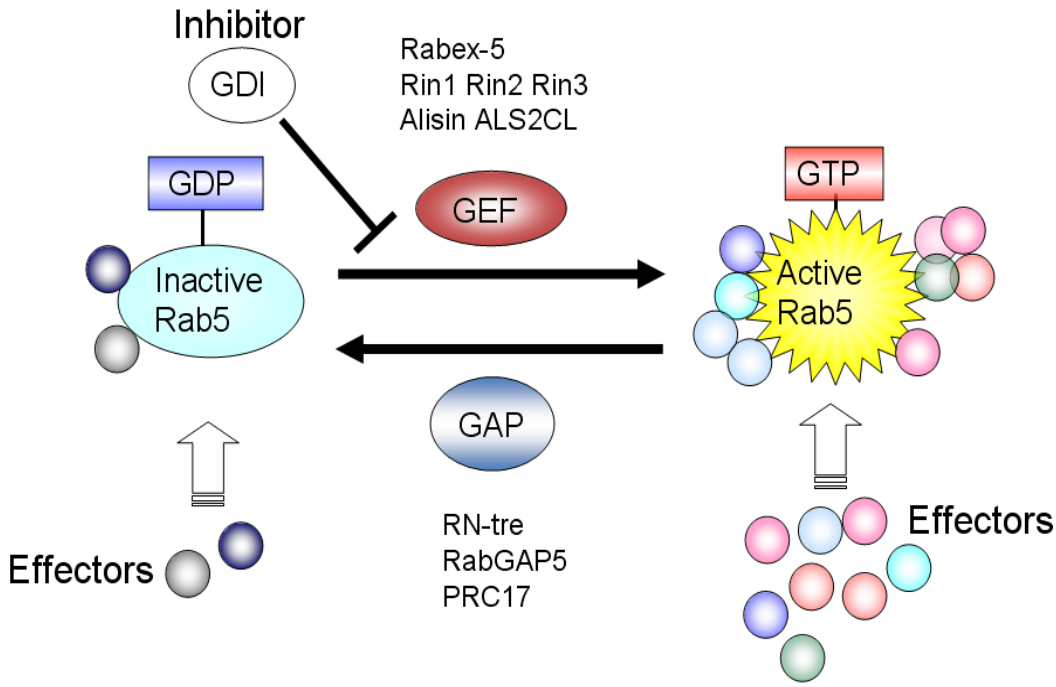

Figure 1. Schematic drawing of known Rab5-binding proteins and their roles in controlling its activity.

RAB5 BINDING DOMAIN ON CAVEOLIN-1

Caveolin-1 is divided into three domains: the N terminal region (residues 1-101: NC domain) and the C terminal region (residues 135-178: CC domain) are separated by a hydrophobic transmembrane domain (TM domain). Additionally, the bulk of caveolin-interacting proteins are signaling molecules, and many of them bear a common caveolin-binding motif that is recognized by a 20-amino acid residue domain of the caveolin molecule (residues 82-101; SD domain)(Figure 2 A). In examining the binding site for Rab5 on caveolin-1, we found out that the amino acid regions cav^{1-101}, $cav^{102-134}$, and $cav^{135-178}$ could bind to Rab5, whilst cav^{1-81} did not bind. Since cav^{1-101} includes the SD domain, unlike cav^{1-81}, we deduce that the SD domain participates in binding to Rab5. These data suggest that amino acid residues 82-178 of caveolin-1 are important for the Rab5-caveolin-1 interaction (15).

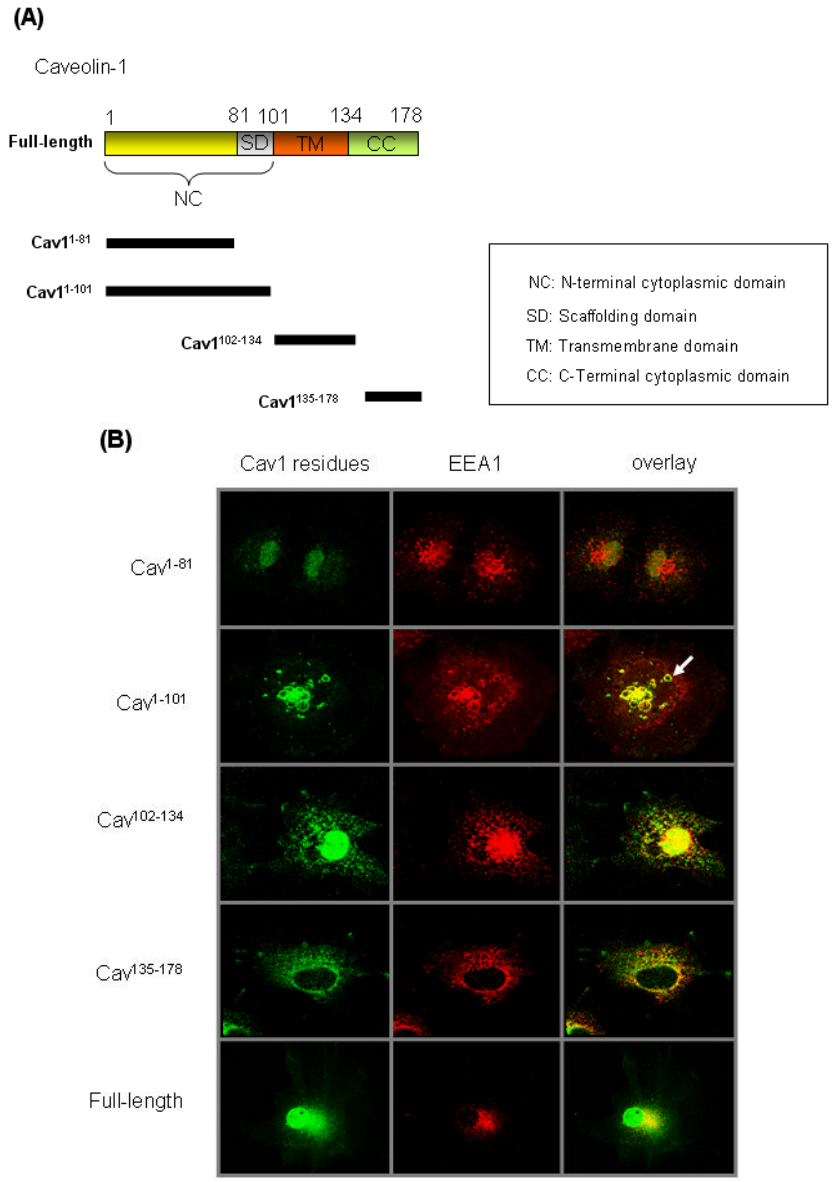

Figure 2. Intracellular localization of caveolin-1 mutants and EEA1. (A) The N-terminal cytoplasmic domain containing the Scaffolding (SD) domain (residues 1-101: cav1-101) and without the SD domain (residues 1-81: cav1-81). The C-terminal cytoplasmic (CC) domain (residues 135-178: cav135-178) is separated from the N-terminal domain by a hydrophobic transmembrane domain (residues 102-134 cav102-134). (B) Each HA-caveolin-1 deletion mutant was expressed in Cos-1 cells. Cells were fixed and stained for HA and EEA1 (early endosome marker) using specific antibodies and observed under a confocal microscope. In brief, cells were fixed with 4% formaldehyde in PBS for 10 min. Nonspecific binding of antibodies was blocked by 5% sheep serum for 60 min, after which cells were incubated with primary antibody in 5% sheep serum for 60 min. Bound primary antibodies were visualized with a secondary antibody. After extensive washing with an ECL wash, slide glasses were mounted with IMMU-Mount (Thermo Scientific, Pittsburgh, PA, USA). Cos-1 cells were observed with confocal fluorescence microscopy (OLYMPUS, FV500-IX). Although Cav^{1-81} did not show any co-localization with EEA-1, $cav1^{1-101}$, $cav^{102-134}$ and $cav^{135-178}$ co-localized with EEA-1. It is noteworthy that when we over-expressed cav^{1-101}, the EEA-1 and cav^{1-101} positive organelles were round-shaped (arrow).

CAVEOLIN-1 AND RAB5 CO-EXIST IN A TUBERIN-POSITIVE COMPARTMENT

TSC1 and *TSC2* function as tumor suppressor genes and, when mutated, they give rise to abnormal cell proliferation and growth. It was previously reported that tuberin, the transcript of *TSC2*, may bind to Rabaptin-5 and control its Rab5 activity (16). This is particularly intriguing since aberrations in the Rab5-dependent endocytic pathways can have profound effects on cell growth and differentiation. These findings led us to hypothesize that tuberin may be associated with certain vesicular or microsomal compartments that participate in trafficking. Our experiments indicated that tuberin-bound fractions contain Rap1, Rab5, and caveolin-1. In brief, when the compartment containing tuberin was immuno-precipitated using IgG-conjugated Dynabeads coated with (+) and without (−) anti-tuberin antibody, we observed both Rab5 and caveolin-1 in this fraction (17). This led us to the hypothesis that the caveolin-1 containing compartment is associated with Rab5 along with tuberin.

RAB5 IS ACTIVATED DURING CAVEOLAE-MEDIATED ENDOCYTOSIS

L. Pelkmans has reported (7) that caveolin-1 dependent endocytosis was stimulated only by constitutively activated Rab5 (Rab5aQ79L) among the other all Rab proteins. Therefore, we next investigated whether Rab5 binds to caveolin-1 after activation, or alternatively if the caveolin-1 interaction activates Rab5. We were also interested in how long the interaction would persist.

In prior experiments, we observed increased CTXB uptake when the binding domain of Rab5 was expressed in the cells (15). Thus we examined whether the caveolin-1 deletion mutants could affect Rab5 activity by using the methods of Liu J. *et al*. (18). The GST-R5BD (Rab5 binding domain) fusion protein was used to pull down Rab5-GTP in cell lysates, followed by immunoblot analysis with a GFP antibody to detect the relative amount of Rab5-GTP. Over-expressing $cav1^{1-101}$, $cav^{102-134}$ and $cav^{135-178}$ increased the Rab5 GTP level, but $cav1^{1-81}$ over-expression had no effect. These data showed for the first time that the Rab5 binding domains of caveolin-1 increase Rab5 activity (15).

THE RAB5 BINDING SITE IS NECESSARY FOR LOCALIZATION TO THE EARLY ENDOSOME BUT NOT TO THE GOLGI APPARATUS

When YFP-tagged dominant-active Rab5 and CFP-tagged caveolin-1 were over-expressed in Cos-1 cells, co-localization of Rab5, caveolin-1 and the early endosome marker, EEA-1, was observed. However, co-localization of these three proteins in the cell did not occur following over-expression of dominant-inactive Rab5 (15). As previously discussed in this section, the amino acids 1 to 101 contain the N-terminal cytoplasmic domain that includes the scaffold domain (SD) domain (cav^{1-101}), whereas amino acids 1-81 lacks the SD domain (cav^{1-81}). Previously we showed that the SD, TM and CC domains interact with Rab5 in Cos-1 cells (15). Thus, we next studied whether the SD, TM and CC domains are necessary for localization of caveolin-1 to the early endosomes. Full-length caveolin-1 showed an

obvious co-localization with EEA-1 (Figure 2 B). As expected, cav^{1-81} did not co-localize with EEA-1, whereas $cav1^{1-101}$, $cav^{102-134}$ and $cav^{135-178}$ did (Figure 2B). It is noteworthy that when cav^{1-101} was over-expressed, the EEA-1 and cav^{1-101} positive organelles were round-shaped. In contrast, over-expression of $cav^{102-134}$ or $cav^{135-178}$ resulted in a more speckle-shaped organelle.

$Cav^{135-178}$ has previously been shown to significantly co-localize with Golgi marker proteins in Cos-7 cells (19). To investigate whether the CC domains are required for Golgi apparatus localization in Cos-1 cells, a deletion mutant was introduced into these cells. In addition to full-length caveolin-1, $cav^{135-178}$ co-localized with Golgi marker GM130, indicating that the CC domain is required for early endosome and Golgi apparatus localization. However, cav^{1-81}, cav^{1-101} and $cav^{102-134}$ did not localize to the Golgi apparatus (Figure 3).

RELATIONSHIP BETWEEN FUNCTIONAL DOMAINS OF CAVEOLIN-1 AND ENDOCYTOSIS

We next examined the connection between the Rab5 binding domain and CTXB uptake. As expected, cav^{1-81} did not show any co-localization with CTXB, whereas $cav1^{1-101}$, $cav^{102-134}$ and $cav^{135-178}$ did (Figure 4). Interestingly, the intracellular co-localization of CTXB and cav^{1-101} resulted in more round-shaped in cells. In contrast, cells showing CTXB and $cav^{135-178}$ co-localization were more peri-nuclear. Additionally, localization of $cav^{102-134}$ was interspersed and co-localized with CTXB. Taken together, these observations suggest that the function of the SD domain during caveolae-mediated endocytosis differs from that of the CC and TM domains.This is to say that the SD domain is necessary to activate the Rab5 and therefore, transport the ligands towards the early endosome. However, for further transport to the Golgi apparatus, the $cav^{135-178}$ is required and this domain is not participating in the Rab5 activation.

ANTICIPATED CAVEOLAE-MEDIATED ENDOCYTOSIS MECHANISM

Our results indicate that caveoli-1 binds only to the activated form of Rab5. Activation of Rab5 is usually mediated by GDP–GTP exchange factors (GEFs), Rabex-5, Rin1, Rin2, Rin3, Alsin and ALS2CL, which generate the Rab5-GTP complex (20). These proteins all contain a specific, highly conserved domain (Vps9 domain) that catalyzes nucleotide exchange on Rab5. However, no obvious Vps9 domain was found on caveolin-1, so one may therefore speculate that caveolin-1 may recruit GEF or maintain its activity through direct binding. Indeed, we observed enlarged endosomes similar to those produced by expressing dominant-active Rab5 when caveolin-1 was introduced to 293T cells, which show high caveolin-1 expression (21). Moreover, rabaptin-5 has been reported to interact with tuberin, and we observed that caveolin-1 and Rab5 co-exist in the tuberin-positive compartment. Thus, one may speculate that rabaptin-5 may control rab5 activity through tuberin located on the caveolae and/or caveosome.

On the other hand, in considering the results of experiments with caveolin-1, treatment of cells with pervanadate or vanadate can induce phosphorylation of caveolin-1, and it internalizes from the cell surface (22). The internalized caveolin-1 co-localized with endocytosed transferrin in the Rab5-positive compartment, where caveolin-1 was phosphorylated (22). Thus phosphorylation of caveolin-1 may also play a role in Rab5 activation.

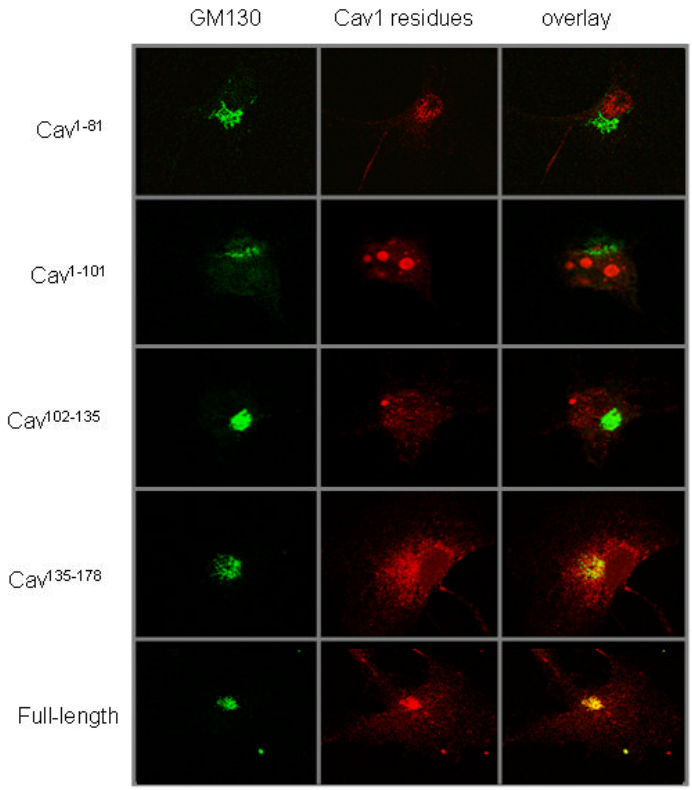

Figure 3. Intracellular localization of caveolin-1 mutants and Golgi apparatus. Each HA-caveolin-1 deletion mutant was expressed in Cos-1 cells. Cells were fixed and stained for HA and GM130 (Golgi apparatus marker) using specific antibodies and observed under a confocal microscope. Although cav1$^{1-101}$, cav$^{102-134}$ and cav$^{135-178}$ co-localized with EEA-1 (Figure 2 B), they did not co-localize with Golgi marker GM130. However, Cav$^{1-81}$ did co-localize with the Golgi marker GM130.

Finally, when we looked at the intracellular localization of caveolin-1 domains, we found that this domain is involved in Rab5 activation. Previous experiments using dominant-negative caveolin-1, which contains a dysfunctional SD domain, revealed the importance of the SD domain during transportation from caveolae to the early endosomes (23). This experiment by Querbes *et al*. indicated that expression of this mutant did not inhibit caveolae sorting from the early endosome to the Golgi apparatus. Indeed, our study showed that when cav$^{1-101}$ was over-expressed, the enlarged early endosome was observed even in the absence of dominant-active Rab5 (Figure 2) and CTXB in the enlarged endosome (Figure 4). In addition, cav$^{1-101}$ did not localize to the Golgi apparatus (Figure 3). Taken together, these data suggest that the SD domain can control the early stage of caveolae-mediated endocytosis, but

it may not be involved in transportation from the early endosome to the Golgi apparatus (Figure 5)

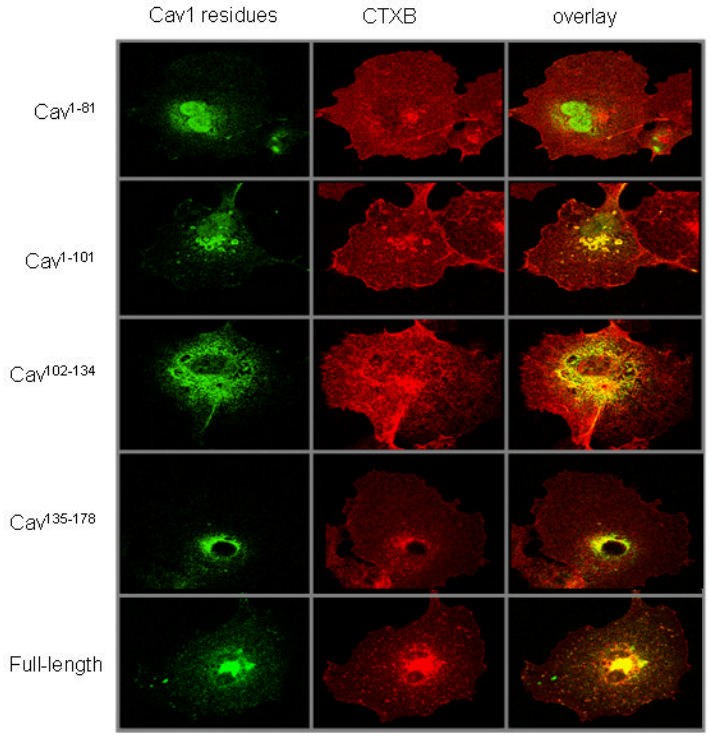

Figure 4. Functional analysis of distinct caveolin-1 domains by CTXB internalization. (A) Each HA-caveoli-1 deletion mutant was introduced into Cos-1 cells and pre-incubated with CTXB at 10°C. After removing the CTXB, cells were further incubated for 20 min at 37°C. Cells were fixed and stained for HA using a specific antibody and observed under a confocal microscope. Although Cav^{1-81} did not show any co-localization with CTXB, $cav1^{1-101}$, $cav^{102-134}$ and $cav^{135-178}$ co-localized with CTXB. Interestingly, the intracellular co-localization of CTXB and cav^{1-101} was associated with a more round-shaped configuration. In contrast, cells with CTXB and $cav^{135-178}$ co-localization appeared more peri-nuclear.

CONCLUSION AND REMARKS

In conclusion, we have demonstrated that Rab5 and the caveolin-1 complex participate in trafficking from caveolae on the plasma membrane to early endosomes. Moreover, the SD domain of caveolin-1 strongly localizes to early endosomes. Thus, our results provide important new insight into the roles of Rab5 and caveolin-1 during caveolae-mediated endocytosis (Figure 5).

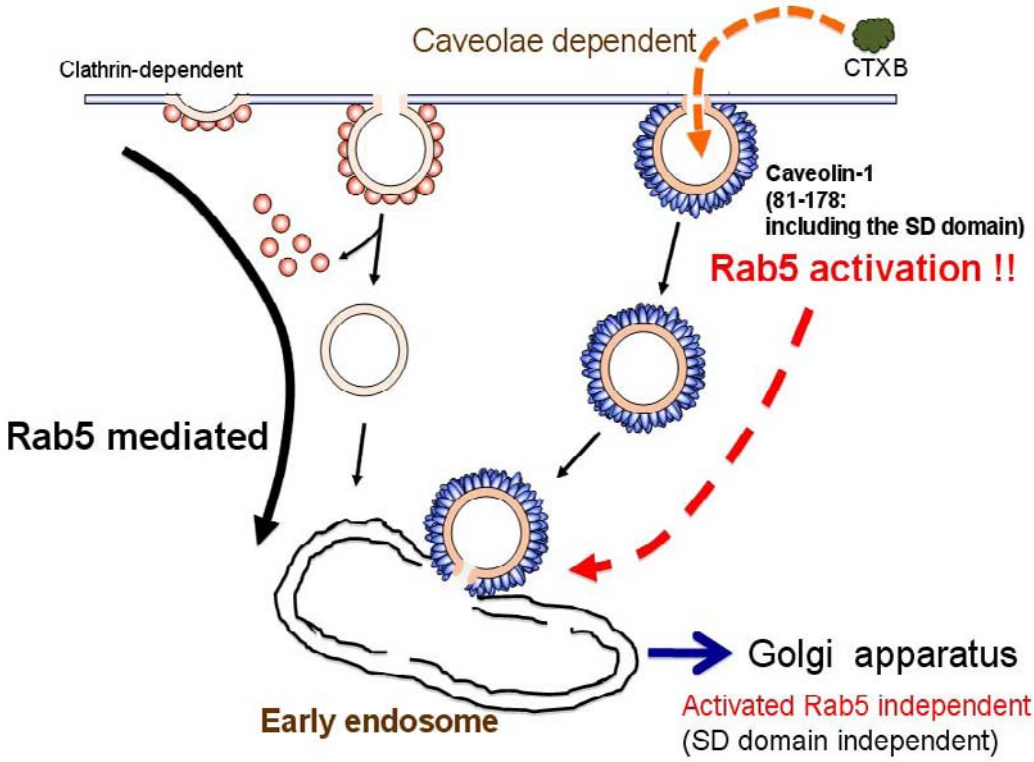

Figure 5. Schematic drawing of caveloin-1 mediated internalization via rab5 activation.

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ABBREVIATIONS

CC domain	C-terminus domain
SD domain	Scaffolding domain
TM domain	transmembrane domain
CTXB	Cholera Toxin subunit B

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Chapter 12

ENDOCYTIC DYSFUNCTYION AND ALZHEIMER'S DISEASE

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ABSTRACT

Although the extracellular deposition of β-amyloid protein (Aβ) as senile plaques (SPs) is an invariable pathological feature of Alzheimer's disease (AD), recent studies suggest that the accumulation of intracellular Aβ may represent an early event in the pathogenesis of AD. In the case of familial AD, evidently the expression of causative genetic mutations likely enhances Aβ generation, which can cause buildup of intracellular Aβ. However, how intracellular Aβ accumulates in sporadic AD, a major form of the disease, remains to be clarified.

Recently, growing evidences suggest that endocytic dysfunction is involved in AD pathology. In brains of early stage AD patients, neuronal endocytic pathology such as the accumulation of β-amyloid precursor protein (APP) in enlarged early endosomes is observed even before SP deposition. Moreover, endocytic dysfunction induces Aβ accumulation in endosomal compartments. Aβ is produced from APP through sequential proteolytic cleavages by β- and γ-secreatases, and such amyloidogenic cleavage of APP can occur through the endocytic pathway. These findings suggest that endocytosis is involved in APP metabolism itself and that endocytic dysfunction may cause the accumulation of intracellular Aβ. In this review, I summarize findings at present and discuss the hypothesis that endocytic dysfunction may underlie AD pathology.

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SHORT COMMUNICATION

Alzhemier's disease (AD) is a progressive neurological disorder causing dementia, one of the biggest problems in elderly people. Although the extracellular deposition of aggregated β-amyloid pepteide (Aβ) as senile plaque (SP) is one of the pathological features of AD (45), accumulating evidences suggest that intracellular Aβ accumulation would be responsible for AD pathology (21). However, it has been controversial where Aβ is produced from its precursor protein, APP, in neurons. Because APP and the secretases required for Aβ production are frequently observed in secretary pathway such as ER and Golgi, Aβ production is considered to occur in trans-Golgi network (TGN) (11, 26, 40, 49). Recently, on the other hand, growing evidences suggest that APP metabolism is mediated by endocytosis (4, 6, 32). Although Aβ is produced from APP through sequential proteolytic cleavages by β- and γ-secreatase (4, 6), APP can be alternately cleaved by α-secretase within the Aβ domain, and the α-site cleavage is considered to occur at plasma membranes (18, 48). This finding suggests that APP can move onto plasma membrane after its secretion from TGN. Moreover, recent studies confirmed that both APP and Aβ are localized to endosomes (6, 31). Taken together, these findings suggest that Aβ can be produced not only in TGN but also in endocytic pathway, and currently, such β-site cleavage is considered to occur mainly in endocytic pathway (4, 6). Moreover, much importantly, recent findings suggest that endocytic dysfunction may be involved in AD pathology itself.

Endocytosis is the process required for the uptake of nutrients, immune response, the regulation of cell surface receptor signaling, and so on (36). Vesicle cargo, or simply "cargo", is internalized via clathrin-dependent or independent pathways, and then fused with early endosomes (23) (Figure 1). Early endosomes can be recycled back to the plasma membrane directly, and also sorted to the late recycling compartment or late endosomes, leading to the fusion with lysosomes for degradation (23) (Figure 1). Endosome trafficking is coordinated by a small molecular weight G-proteins called as Rabs (28, 53). Rab proteins are members of the Ras superfamily of GTPases that cycle between GTP-bound active and GDP-bound inactive states. Each Rab protein regulates distinct endosome trafficking steps temporally and spatially by facilitating vesicle motility, tethering, and fusion (22, 42) (Figure 1). Drug treatments by using chloroquine or ammonium chloride are often used to disturb endosome trafficking experimentally, and such biochemical analyses revealed that endocytic dysfunction can cause the upregulation of Rab GTPases and enlargement of endosomes (34). Evidently, Cataldo et al confirmed that endocytic pathology such as abnormally enlarged endosomes is frequently observed in early stage of AD patient brains, and APP accumulates in those enlarged early endosomes (4, 5, 6, 39). On the other hand, Cataldo et al also demonstrated that the upregulation of Rab causes enlargement of endosomes, resulting in the retardation of endosome trafficking (7). Moreover, they also observed endocytic pathology in fibroblasts derived from Down syndrome patients that has also Aβ pathology due to trisomic APP, and Rab expression level is clearly increased (7). Thus the endocytic pathology is really related to endocytic dysfunction, and it may be responsible for Aβ pathology. Besides APP metabolism itself, Yuyama et al demonstrated that endocytic dysfunction induces GM1 ganglioside accumulation, leading to assemble of GM1 ganglioside-bound Aβ, the seed molecule for Aβ aggregation (50-52).

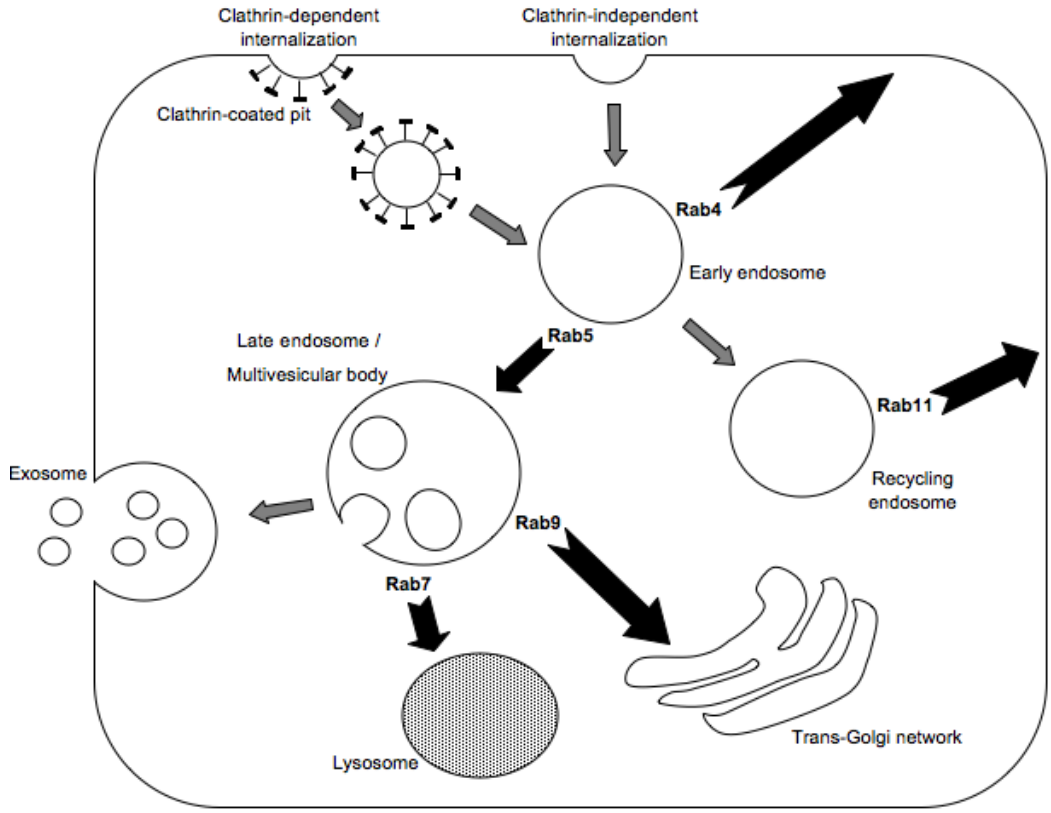

Figure 1. Simplified schema of endocytic pathway and major Rab GTPases. Each Rab protein regulates distinct endosome trafficking steps (black solid arrows). Rab4 mediates direct recycling traffic from early endosome. Rab5 mainly regulates earky endosome trafficking toward late endosome / multivesicluar body. Rab7 mediates late endosome trafficking and its fusion with lysosome. On the other hand, Rab9 mediates the membrane trafficking from late endosome back to trans-Golgi network. Rab11 regulates long-range recycling traffic.

It is undoubted that aging is the biggest risk factor for AD, however, it remains uncertain how aging causes endocytic dysfunction. Axonal transport is the bi-directional intracellular transport system, mainly carried out by two well-known motor protein complexes such as kinesin and dynein (3, 35, 41, 47). Cytoplasmic dynein is a microtubule-based motor protein required for minus end-directed axonal transport (8, 9). Dynactin, another microtubule-associated protein, binds to dynein intermediate chain (DIC) via its subunit, P150glued/dynactin (DYN) to form dynein-dynactin complexes that mediate minus end-directed vesicle transport, which includes endosome trafficking (10, 12-14, 16, 17). We have previously shown that the interaction between DIC and DYN is clearly attenuated in aged monkey brains, suggesting that aging may impair dynein-mediated transport (29), and other studies also support this idea (21, 24, 25, 27). Noteworthy, our recent study demonstrated that siRNA-induced dynein dysfunction reproduced endocytic pathology such as APP accumulation in enlarged early endosomes, and dynein dysfunction also induced intracellular accumulation of Aβ (30). These findings suggest that age-dependent impairment of dynein-

mediated transport may be responsible for age-related endocytic dysfunction, and even for Aβ pathology itself.

Endosome trafficking is involved in autophagy machinary, the bulk degradation system by delivering cytoplasmic substrates for lysosomal degradation pathway. Macroautophagy (referred to here as autophagy) is a process in which cells form double-membrane vesicles, called as autophagosome, and the autophagosome fuses with lysosome for degradation of its contents (15, 33, 37, 38). Autophagosome can fuse with endosomes to form amphisomes (2). Although it remains unclear whether amphisome formation is indispensable for autophagosome-lysosome fusion, it is reasonable idea that endocytic dysfunction can subsequently interrupt autophagosome clearance. Moreover, because the fusion of autophagosome with lysosome is also mediated by dynein (19, 43, 44), age-dependent dynein dysfunction causes not only endocytic dysfunction but also the breakdown of autophagy. Autophagy is induced under the certain stress condition such as starvation, and also important for the clearance of misfolded or aggregated proteins (43, 44). Thus, although additional investigations are needed, dynein dysfunction may be involved in not only AD pathology but also other neurodegenerative disorders characterized by the abnormal intracellular accumulation of causative proteins.

Endocytosis system is also involved in neurotransmission (1, 20, 46), and then endocytic dysfunction can cause the impairment of synaptic activity, resulting in cognitive impairment. Moreover, the glial response against Aβ toxicity such as Aβ uptake is also mediated by endocytosis. Altogether, this line of studies focusing on endocytosis would greatly contribute to understand the mechanism of AD pathology, and intracellular transport system may represent a prime target for the development of new therapeutics.

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