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G. Chinnadurai

CtBP Family Proteins

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G. Chinnadurai
Institute for Molecular Virology
Saint Louis University Health Sciences Center
St. Louis, Missouri, U.S.A.

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EDITOR

G. Chinnadurai
Institute for Molecular Virology
Saint Louis University Health Sciences Center
St. Louis, Missouri, U.S.A.
Email: chinnag@slu.edu
Chapter 1

CONTRIBUTORS

Hitoshi Aihara
Department of Cell
and Developmental Biology
Weill Medical College
of Cornell University
New York, New York, U.S.A.
Chapter 2

Claire Baker
Cancer Research UK Institute
for Cancer Studies
University of Birmingham
Birmingham, U.K.
Chapter 5

Paola M. Barral
Cancer Research UK Institute
for Cancer Studies
University of Birmingham
Birmingham, United Kingdom
Chapter 5

Johann Helmut Brandstätter
Department of Neuroanatomy
Max Planck Institute for Brain Research
Frankfurt/Main, Germany
and
Institute for Biology
University of Erlangen-Nürnberg
Erlangen, Germany
Chapter 11

Rachel K. Bruton
Cancer Research UK Institute
for Cancer Studies
University of Birmingham
Birmingham, United Kingdom
Chapter 5

Daniela Corda
Department of Cell Biology
and Oncology
Consorzio Mario Negri Sud
Santa Maria Imbaro (Chieti), Italy
Chapter 10

Merlin Crossley
School of Molecular
and Microbial Biosciences
University of Sydney
New South Wales, Australia
Email: m.crossley@mmb.usyd.edu.au
Chapter 3

Susanne tom Dieck
Department of Neuroanatomy
Max Planck Institute for Brain Research
Frankfurt/Main, Germany
Chapter 11

Clark C. Fjeld
Vollum Institute
Oregon Health and Science University
Portland, Oregon, U.S.A.
Chapter 7

Steven M. Frisch
Mary Babb Randolph Cancer Center
and Department of Biochemistry
West Virginia University
Morgantown, West Virginia, U.S.A.
Email: sfrisch@hsc.wvu.edu
Chapter 4

Philip H. Gallimore
Cancer Research UK Institute
for Cancer Studies
University of Birmingham
Birmingham, United Kingdom
Chapter 5

Roger J.A. Grand
Cancer Research UK Institute
for Cancer Studies
University of Birmingham
Birmingham, United Kingdom
Email: R.J.A.Grand@bham.ac.uk
Chapter 5

Richard H. Goodman
Vollum Institute
Oregon Health and Science University
Portland, Oregon, U.S.A.
Chapter 7

Cristina Hidalgo Carcedo
Department of Cell Biology
and Oncology
Consorzio Mario Negri Sud
Santa Maria Imbaro (Chieti), Italy
Chapter 10

Jeffrey D. Hildebrand
University of Pittsburgh
Department of Biological Sciences
Pittsburgh, Pennsylvania, U.S.A.
Email: jeffh@pitt.edu
Chapter 6

James R. Lundblad
Division of Molecular Medicine
Department of Medicine
and Biochemistry and Molecular Biology
Oregon Health & Sciences University
Portland, Oregon, U.S.A.
Email: lundblad@ohsu.edu
Chapter 9

Yutaka Nibu
Department of Cell and Developmental
Biology
Weill Medical College of Cornell
University
New York, New York, U.S.A.
Email: yun2001@med.cornell.edu
Chapter 2

Amanda C. Nottke
Vollum Institute
Oregon Health and Science University
Portland, Oregon, U.S.A.
Chapter 7

Julian Parkhill
The Sanger Institute
Wellcome Trust Genome Campus
Hinxton, Cambridge, U.K.
Chapter 5

Jose Perdomo
Centre for Thrombosis and Vascular
Research
School of Medical Sciences
and Department of Medicine
St. George Clinical School
University of New South Wales
New South Wales, Australia
Chapter 3

Lorena Perrone
Department of Cell and Developmental
Biology
Weill Medical College of Cornell
University
New York, New York, U.S.A.
Chapter 2

Frank Schmitz
Institute for Anatomy and Cell Biology
University of the Saarland
Homburg, Germany
Chapter 11

Yang Shi
Department of Pathology
Harvard Medical School
Boston, Massachusetts, U.S.A.
Email: yang_shi@hms.harvard.edu
Chapter 8

Yu-Jiang Shi
Department of Pathology
Harvard Medical School
Boston, Massachusetts, U.S.A.
Chapter 8

Stefania Spanò
Department of Cell Biology
and Oncology
Consorzio Mario Negri Sud
Santa Maria Imbaro (Chieti), Italy
Chapter 10

Tadge Szestak
The Babraham Institute
Babraham Research Campus
Cambridge, U.K.
Chapter 5

Hirokazu Tsukaya
Okazaki Institute for Integrative
Bioscience
National Institute for Basic Biology
Myodaiji-cho, Okazaki
and
Japan Graduate University
for Advanced Studies
Kanagawa, Japan
and
Graduate School of Science
Kyoto University
Kyoto, Japan
Email: tsukaya@nibb.ac.jp
Chapter 12

Alexis Verger
School of Molecular and Microbial
Biosciences
University of Sydney
New South Wales, Australia
Email: a.verger@mmb.usyd.edu.au
Chapter 3

Qinghong Zhang
Vollum Institute
Oregon Health and Science University
Portland, Oregon, U.S.A.
Email: zhangq@ohsu.edu
Chapter 7

PREFACE

When I received an invitation from Ron Landes (Landes Bioscience) to edit a book on CtBP family proteins, I was gratified to realize that the importance of these proteins has reached the level of deserving a 'separate' book. As the reader can see, there has been significant advancement in our understanding of the functions of these proteins in the past ten years since CtBP1 was cloned in our laboratory. Genetic and biochemical studies with *Drosophila* provided the critical evidence to show that dCtBP is a transcriptional corepressor. Genetic studies with mutant mice have established that these proteins are essential for animal development. The CtBP family proteins are unique in several aspects. They were the first among proteins containing a metabolic enzyme fold to be implicated in transcriptional regulation. The vertebrate CtBPs exhibit distinct nuclear and cytosolic activities. The crystal structures of CtBP1 and molecular modeling studies have illuminated the molecular basis of its dual activity and the interaction with target peptides. The organization of the vertebrate CtBP2 gene has provided a novel example of genomic consolidation indicating how a single gene could code for two diverse proteins. I believe that this book will be a valuable reference source for new researchers to understand more about the CtBP family proteins and their role in growth, development and oncogenesis. It may also serve the needs of researchers who already are active in this field by providing a single extensive reference source. In the first chapter, I have attempted to provide a comprehensive general review of CtBP family proteins and refer to the individual chapters for more detailed discussions. I hope that this book will stimulate more interest and pave the way for additional discoveries on the functions of these fascinating proteins.

I greatly appreciate the various contributors who provided insightful discussions on various topics. I wish to thank three former and present members of our laboratory who were instrumental in identification and cloning of the founding member of the CtBP family proteins. The work of T. Subramanian led to the discovery that the C-terminal region of the adenovirus E1A oncogene restrains the oncogenic activity of the activated Ras oncogene. The patience and perseverance of Janice Boyd led to the identification of the CtBP phosphoprotein. The hard work and dedication of Ute Schaeper led to the cloning of the CtBP1 cDNA. I am thankful to Catherina Svensson (Uppsala University), who shared the 'growing pains' of the early days of CtBP through her discussions. The initial functional insight that CtBP might be a transcriptional regulator came from her work. Ling-jun Zhao provides me with a constant source of energy and inspiration with his insightful discussions on CtBPs. I wish to express my gratitude to Ron Landes for the invitation to edit this book, and to Cynthia Conomos and Kristen Shumaker for their help in coordinating this publication project.

G. Chinnadurai

CHAPTER 1

CtBP Family Proteins:
Unique Transcriptional Regulators in the Nucleus with Diverse Cytosolic Functions

G. Chinnadurai*

Abstract

CtBP family proteins are unique in animals and in plants. The invertebrates and plants contain a single CtBP family gene while vertebrates have two genes. Genetic studies in *Drosophila* and in mice indicate that CtBPs play pivotal roles in animal development. The vertebrate CtBPs (CtBP1 and CtBP2) are highly related and are functionally redundant for certain developmental processes and non redundant for others. The vertebrates code two isoforms of each CtBP1 and CtBP2. The animal CtBPs exhibit a highly conserved sequence and structural similarity to D-isomer specific 2-hydroxy acid dehydrogenases (D2-HDH). Structural and molecular modeling studies indicate that CtBP1 is a dehydrogenase and could also bind with acyl-CoA under a different configuration. The CtBP family members function predominantly as transcriptional corepressors in the nucleus in conjunction with a number of different DNA binding repressors. The transcriptional regulatory activity of CtBPs appears to be regulated by NAD(H)-binding and the metabolic status of the cell. The corepressor complex of CtBP1 contains enzymatic constituents that mediate coordinated histone modification by deacetylation and methylation of histone H3-K9 and demethylation of histone H3-K4. In the cytosol, they perform diverse functions associated with membrane trafficking, central nervous system synapses and in regulation of the microtubule cytoskeleton. The mammalian CtBPs modulate oncogenesis by regulating the activities of tumor suppressor genes and cellular and viral oncogenes, consistent with a role in tumor suppression as well as in tumor promotion. The CtBPs promote tumorigenesis by repressing transcription of several critical pro-apoptotic genes and by inhibiting genes involved in the regulation of epithelial to mesenchymal transition. This Chapter presents a comprehensive general review of the CtBP field and highlights contents of the individual Chapters of this book which contain detailed discussions on structure and functions of animal and plant CtBP family proteins.

Introduction

CtBP (C-terminal binding protein) was identified in 1993 as a 48 kD cellular phosphoprotein that bound to the C-terminal region of the adenovirus E1A oncoprotein.[1] In 1995, the cDNA for the founding member of the CtBP family protein was cloned and the encoded protein was shown to bind to a five amino acid motif (PLDLS) conserved at the C-terminus of E1A of all primate adenoviruses.[2] The CtBP protein originally identified as the E1A-binding protein is now known as CtBP1. Subsequently, a highly homologous human protein termed CtBP2 was

*G. Chinnadurai—Institute for Molecular Virology. Saint Louis University Health Sciences Center. 3681 Park Avenue, St. Louis, Missouri 63110, U.S.A. Email: chinnag@slu.edu

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identified by analysis of EST data bank sequences[3] and mouse CtBP2 was cloned by a two hybrid screen against the transcription factor BKLF.[4] The initial amino acid homology searches revealed that CtBP1 shared a striking homology to D-isomer specific 2-hydroxy acid dehydrogenases (D2-HDH).[2] The interaction between a cellular protein with a metabolic enzyme fold and the E1A viral oncoprotein was unexpected since E1A functions primarily as a transcriptional modulator (reviewed by Gallimore and Turnell).[5] However, a possible role of CtBP in transcriptional repression was soon suggested by a tethering transcriptional assay.[6] In these assays, the N-terminal conserved region (CR1) of E1A fused to a heterologous DNA-binding domain (Gal4) strongly activated a synthetic promoter containing a Gal4 binding site. The CR1 region of E1A contains the sequences for interaction with a SWI/SNF-related chromatin remodeling complex, TRRAP/p400[7-9] and also the binding sites for the nuclear acetylase P/CAF.[10] Inclusion of the C-terminal region of E1A in the chimeric Gal4-E1A construct abrogated CR1-mediated transcriptional activation. Deletion of the CtBP-binding motif relieved the repressive activity of the C-terminal region. These results suggested that interaction of CtBP with the C-terminal region antagonized the trans-activation activity of CR1 in *cis*.

A definitive role for CtBP in transcriptional repression became evident with the identification and cloning of the *Drosophila* homolog of CtBP (dCtBP) by the laboratories of Michael Levine[11] and Susan Parkhurst.[12] Since then, a large number of DNA-binding transcriptional repressors have been reported to recruit CtBPs via the PLDLS-related binding sites.[13,14] The studies with dCtBP and a number of subsequent studies with vertebrate CtBP1 and CtBP2 have established that CtBPs function predominantly as transcriptional corepressors. However, splice variants of the vertebrate CtBPs have been shown to be involved in unrelated biological processes in the cytosol. During the past ten years since the cloning of CtBP1, there has been a substantial increase in our understanding of the structure, functions, and mechanisms of action of CtBP family proteins and their role in various biological processes. These advancements include elucidation of the structural determinants of CtBP1 and the molecular basis of its interaction with the CtBP-binding motif and the determination of the roles of CtBP1 and CtBP2 in mouse development. Additionally, several nuclear cofactors that mediate the transcriptional regulatory activity of CtBPs and the CtBP-target genes have been identified. This Chapter will highlight the salient aspects of CtBP family proteins while more detailed discussions can be found in the individual Chapters of this book.

CtBP Family Proteins

The CtBP family proteins are highly conserved in higher eukaryotes. The genomes of invertebrates such as *Drosophila* and *C. elegans* contain a single *CtBP* gene. However, they code different isoforms as a result of differential RNA processing. For example, in *Drosophila* there appears to be at least three different alternatively spliced transcripts of *dCtBP*[15] (see Chapter by Aihara, Perrone and Nibu). The vertebrate genomes contain two different genes, *CtBP1* and *CtBP2* that code for two highly related proteins. The *CtBP1* gene is located on chromosome 4 of humans and on chromosome 5 of mice. In mammals, the *CtBP1* gene expresses two major transcripts as a result of alternate RNA splicing. These transcripts encode two isoforms of CtBP1, which are identical except for a thirteen amino acid region at the N-terminus (Fig. 1A). The short version of CtBP1 (CtBP1-S) corresponds to an isoform designated as CtBP3/BARS[16] (see Chapter by Spano, Hidalogo Careedo and Corda; the designation CtBP3 has now been changed to CtBP1-S). The transcript for CtBP1-S has an alternate inframe exon (exon 2) in the 5'-region which codes for the N-terminal two amino acids while translation of CtBP1-L is initiated from exon 1 of the shorter transcript (Fig. 1A). A fraction of CtBP1 cDNAs also contains an insertion of a codon for a Ser residue (at position 380 in CtBP1-L and at position 369 in CtBP1-S), which also appears to be the result of alternate RNA processing. The functional significance of the extra Ser residue is not known at present. The CtBP1 proteins are concentrated in the nucleus with significant amounts in the cytosol.

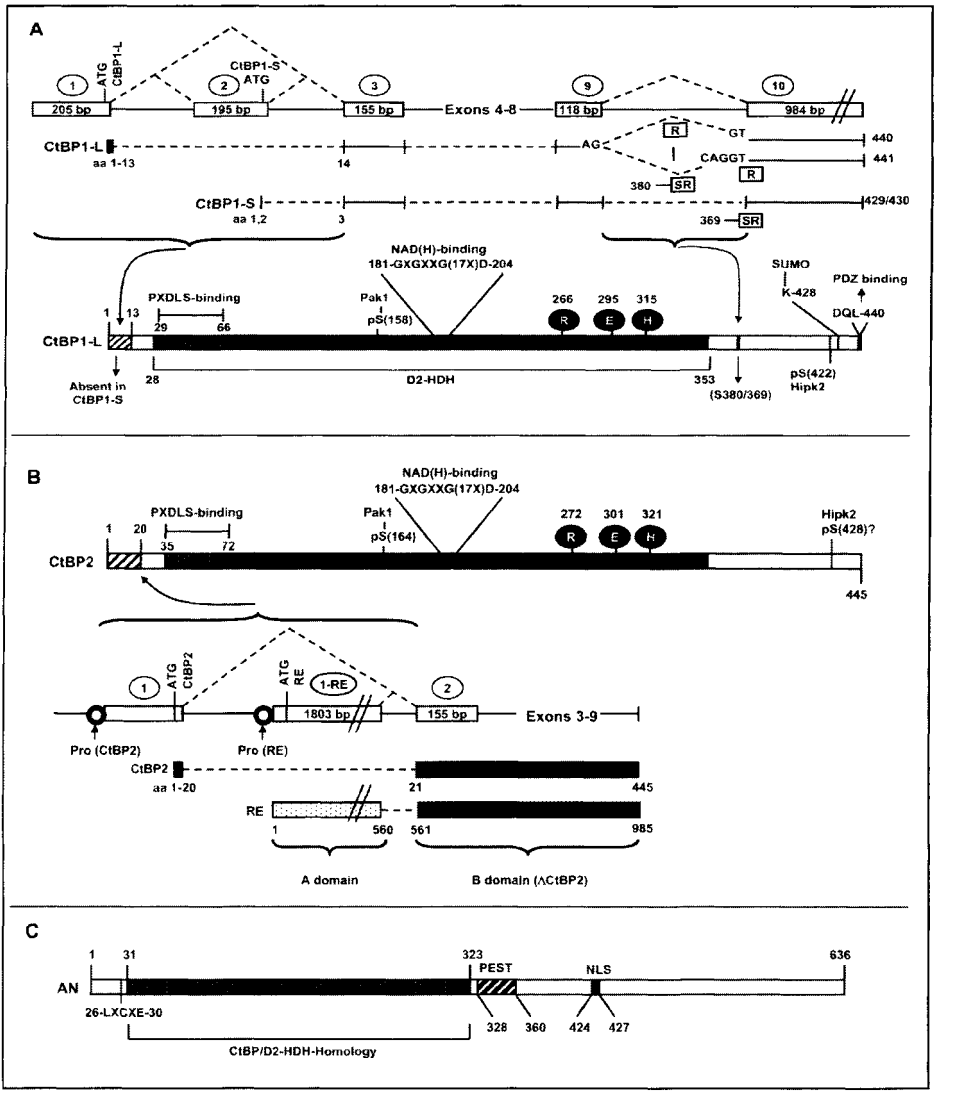

Figure 1. CtBP family proteins. A) Human CtBP1 isoforms. The splicing patterns of CtBP1 transcripts at the 5'- (exons 1 to 3) and 3'- (exon 9 and 10) regions are shown at the top. The intron-exon boundaries are based on NCBI 'Spidey' analysis of CtBP1 reference sequences for variant 1 (gi:61743965) and variant 2 (gi:12746589). The amino acid sequence variations at the N-terminal regions of CtBP1-L and CtBP1-S as well the polymorphic Ser residue (#380/369) are indicated. The domain structure of CtBP1-L is shown at the bottom. The phosphorylation sites by Pak1 and Hipk2, sumoylation site, PXDLS-binding region, and the PDZ-binding regions are indicated. The amino acid residues that constitute the catalytic triad of D2-HDH are indicated within filled circles. The amino acid sequence between residues 28 and 353 is highly conserved among animal CtBPs and D2-HDHs. B) Human CtBP2 isoforms. The domain structure of CtBP2 is shown at the top. The 5' regions of the two transcripts of the *CtBP2* gene are shown at the bottom. The indicated splicing patterns (NCBI, Spidey) of the two transcripts are based on reference sequences, gi: 4557498 (variant 1) and gi: 12746589 (variant 2). The two promoters of the *CtBP2* gene are indicated as 'Pro'. RE indicates RIBEYE. C) Domain structure of ANGUSTIFOLIA (AN) of *Arabidopsis thaliana*. The region of homology shared with animal CtBPs and D2-HDHs as well as other potential sequence motifs are indicated.

The *CtBP2* gene maps to chromosome 10 of humans and in chromosome 7 of mice. The *CtBP2* gene also codes for two protein isoforms (Fig. 1B). The ubiquitously expressed isoform commonly referred to as CtBP2 (48 kD) is highly related to CtBP1. The second isoform, designated as RIBEYE is a 120 kD protein and is predominantly expressed in sensory neurons.[17,18] The two protein isoforms of the *CtBP2* gene are coded by two transcripts that are transcribed from two distinct promoters and contain two different 5'-coding exons.[17] The first coding exon of the *CtBP2* transcript codes for the N-terminal 20 amino acids of CtBP2 while the first coding exon of the *RIBEYE* transcript (located within the first intronic region of the *CtBP2* locus) codes for a large N-terminal domain (designated A-domain) of RIBEYE. The A-domain of RIBEYE is unrelated to other proteins and the B-domain is identical to CtBP2 (aa 21 to 445). RIBEYE lacks the N-terminal 20 amino acid domain of CtBP2 and is localized to the cytoplasm.[19] In contrast, CtBP2 is highly concentrated in the nucleus. Nuclear acetylation by p300 contributes to nuclear retention of CtBP2[19a]. The *CtBP2* gene is unique since its transcription is controlled by two distinct promoters to generate transcripts for two different protein isoforms. In contrast to mammals, the telocast fish express two different RIBEYE proteins coded by two different *CtBP2* genes.[20] The RIBEYE protein, in concert with other protein factors which includes CtBP1, plays a central role in ribbon synapses.

The genomes of terrestrial plants also code for a CtBP family member, ANGUSTIFOLIA (AN). The *AN* gene was first identified in *Arabidopsis thaliana*[21,22] and this gene controls polarity-dependent leaf cell expansion, possibly through controlling the arrangement of the microtubule cytoskeleton (see Chapter by Tsukaya). The N-terminal half of the AN protein shares amino acid sequence homology with animal CtBP family members. Although the sequence conservation between AN and animal CtBPs is not as extensive as among the animal CtBPs, the shared homology between AN and CtBPs is significant and warrants inclusion of AN in the CtBP family. However, some differences between AN and animal CtBPs are evident. In spite of a general sequence similarity to D2-HDH, the AN protein lacks amino acid residues important for the D2-HDH catalytic function and also lacks the consensus NAD(H)-binding motif. In contrast to animal CtBPs, the AN protein of *A. thaliana* does not appear to bind to the prototypical PLDLS motif containing protein E1A (see Chapter 12). Plant AN proteins contain a conserved Rb-binding motif at the N-terminal region. However, it is not known if AN complexes with Rb family members. Like animal CtBPs, AN also appears to be both cytoplasmic and nuclear suggesting that it functions at both locations. Genetic and biochemical studies have indicated an association between the AN protein and the kinesin motor ZWICHEL (ZWI) consistent with a role for AN in control of the microtubule cytoskeleton.[21] Gene expression profiling studies suggest that AN might also function as a transcriptional corepressor since expression of a set of genes was elevated in *an* mutant plants.[22] Since some functionally deficient *an* mutations are located in the C-terminal unique region of the *AN* gene (not present in animal CtBPs), it appears that AN may have functions in addition to those that are controlled by the CtBP-homology region. Results from future studies are eagerly awaited to determine if the CtBP-homology region of AN can be substituted by animal CtBP sequences.

Nuclear Functions

Transcriptional Repression

The vertebrate CtBPs[4,6,23-25] and the *Drosophila* homolog, dCtBP[11,12] function as transcriptional corepressors in the nucleus. It is now well established that a large number of DNA-binding transcriptional repressors mediate their activity by recruiting CtBP through sequence motifs that resemble the adenovirus E1A CtBP-binding motif, PLDLS.[13,14] Initial studies with *Drosophila* embryos provided strong evidence that dCtBP is a transcriptional corepressor.[11] Since then a number of *Drosophila* repressors have been shown to mediate their activity partly or fully in a dCtBP-dependent manner (see Chapter 2 by Aihara, Perrone and Nibu). These conclusions were based on two different approaches. First, in embryos deficient in maternal dCtBP, the activities of several repressors were impaired. Second, in the transgenic

embryos, repressors mutated in the CtBP-binding motif were defective in transcriptional repression. Although dCtBP interacts with a long range repressor, Hairy,[12] it appears that dCtBP may inhibit the repressive function of Hairy by modulating the activity of the corepressor Groucho (Gro) (which contributes to the repressor activity of Hairy).[26] The manifestation of full activity of short range repressors such as Krüppel, Knirps, Snail and Giant requires dCtBP.[11,27] CtBP appears to contribute quantitatively, rather than qualitatively, to the activity of short range repressors such as Knirps.[28] The molecular mechanism by which dCtBP contributes transcriptional repression in conjunction with these repressors remains to be elucidated. Although hCtBP1 associates with type I histone deacetylases (HDAC),[29-31] it is uncertain whether dCtBP associates with HDAC since short range repressors function normally in mutant embryos that are deficient in dHDAC1[32] and are insensitive to trichostatin A.[33] However, a more recent analysis of a protein complex of the short-range repressor Knirps has revealed the presence of Rpd3 and this association requires the CtBP-dependent repression domain of Knirps.[33a] Thus, the possibility that dCtBP-mediated short-range repression requires the HDAC activity warrant further scrutiny.

Mutational studies indicate that the putative D2-HDH activity of dCtBP is not required for transcriptional repression while the NAD(H)-binding activity is required when expressed as the DNA-binding Gal4-dCtBP fusion protein.[15] In addition to the role in short range repression, recent studies with transgenic *Drosophila* embryos have provided strong evidence that dCtBP plays a critical role in repression mediated by the Polycomb group (PcG) proteins.[34,35] The mammalian transcriptional repressor YY1 which shares significant sequence homology to the *Drosophila* PcG protein Pleiohomeotic (PHO) represses PcG-responsive promoters when expressed as a Gal4-YY1 chimeric protein. The YY1-mediated repression is strongly dependent on dCtBP. These studies have revealed that dCtBP plays direct role in PcG repression by modulating the DNA-binding activity of Gal4-YY1 as well as by recruiting other PcG factors.

With regard to the subcellular localization of the two vertebrate CtBP proteins, CtBP1 localizes to both the nucleus and the cytoplasm, with enhanced nuclear concentration. In contrast, CtBP2 localizes predominantly to the nucleus. Consistent with the high degree of sequence homology shared by these two proteins, both proteins have been shown to possess transcriptional corepressor activity. The sequence conservation would also suggest that they might mediate the transcriptional repression activity through similar mechanisms. Studies with mutant mice also suggest that the two isoforms have overlapping transcriptional functions.[36] Most of our current knowledge on transcriptional regulation by vertebrate CtBPs is derived from studies using CtBP1 as the model. Although structural studies have established that CtBP1 is a D2-HDH[37,38] and biochemical studies indicate that CtBP1 possess a slow DH activity[29,37,39] (see Chapter by Lundblad), the role of DH activity in transcriptional repression by vertebrate CtBPs is not clear and remains controversial.[4,37,40] A proteomics based analysis of the CtBP nuclear protein complex by Yang Shi and colleagues has illuminated some critical aspects of the corepressor function of CtBP1[29] (see Chapter by Shi and Shi). These studies have identified several chromatin modifying enzymatic constituents associated with the CtBP protein complex, in addition to certain DNA-binding repressors (such as ZEB) that have been previously known to recruit CtBP. The CtBP complex contains class 1 histone deacetylases (HDAC1/2) and histone methylases (G9a and HMTase1) suggesting that CtBP1 contributes to transcriptional repression by coordinate histone modification through deacetylation and methylation (Fig. 2). Studies on the CtBP protein complex have also led to the identification of the first histone demethylase, LSD1 (lysine specific demethylase-1). Additionally, the CtBP complex contains the corepressor CoREST.[41,42] The CoREST protein complex also contains HDAC1/2 and LSD1 (BHC110).[43] It appears that CoREST is the direct binding partner of LSD1.[44,45] In Chapter 8, Shi and Shi suggest that the CoREST repressor complex may be substantially similar to the CtBP complex since they share a number of constituents (such as HDAC1/2 and LSD1), and repress a common set of target genes.

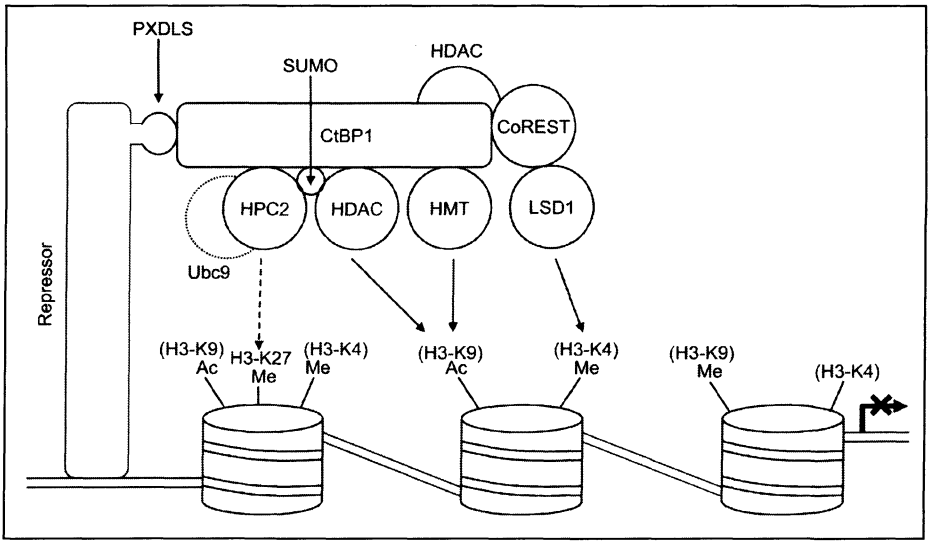

Figure 2. Transcriptional repression by CtBP1. A prototypical DNA-binding repressor (such as ZEB) is depicted to recruit CtBP1 (through the PXDLS motif) and the associated proteins to transcriptionally active areas chromatin in which histone H3 is acetylated (K9) and methylated (K4). During transcriptional repression, the HDACs deacetylate H3-K9 followed by methylation by HMTase. The methylated K4 residue of histone H3 is demethylated by the action of LSD1. It has been suggested that hypoacetylated nucleosomes may be the preferred targets for LSD1,[44] suggesting demethylation of H3-K4 may be a subsequent event after deacetylation of H3-K9. The resulting CH_2O may serve as a potential methyl donor for the action of HMTase.[91] The HPC2 moiety in the CtBP1 complex, in addition to playing a role in sumoylation (by recruiting Ubc9) may also directly interact with the chromatin via methylated H3-K27.[92-94]

The CtBP protein complex also contains the PcG protein HPC2. The interaction between PC2 and CtBP has also been detected previously in two hybrid screenings.[25] The HPC2 protein recruits CtBP1 and Ubc9 to the PcG bodies[46] resulting in sumoylation of CtBP1 at a single Lys (K428) residue.[46,47] The SUMO modification of CtBP1 appears to be critical for its nuclear accumulation. The potential role of HPC2 in CtBP-mediated transcriptional repression remains to be investigated. In Chapter by Shi and Shi, the authors raise the possibility that HPC2 may function by binding to methylated (K27) histone H3 in a fashion analogous to the *Drosophila* Pc protein. Although sumoylation of CtBP1 has been reported to be important for nuclear localization, it is possible that this modification may also play a role in the corepressor activity of CtBP1. Certain transcription factors have been reported to recruit HDACs via the SUMO peptide.[48,49] Additionally, Ubc9 recruited by CtBP1 and HPC2 may also target other transcription factors and histone H4.[50-52] Since the SUMO peptide has been shown to bind HDACs such as HDAC2 and 6 (reviewed by Gill),[53] it would be of interest to determine if the recruitment of HDACs by CtBP1 is dependent on sumoylation. It is also possible that HDAC1/2 may be recruited to the CtBP complex through CoREST. Thus, studies on the CtBP protein complex suggest that coordinate histone modification may be the primary mode of transcriptional repression by CtBP1. However, CtBP1 has also been reported to inhibit the general transcriptional machinery through direct interaction with nuclear acetylases p300 and CBP via a PXDLS motif located within the bromodomain of these enzymes,[54a] as well as through a PXDLS-independent interaction.[55]

As in the case of dCtBP, the dinucleotide binding activity of vertebrate CtBPs also plays an important role in transcriptional activity. The dinucleotides NAD+ and NADH stimulate

dimerization and interaction of CtBP with PXDLS-containing target proteins such as adenovirus E1A.[39,56] Since CtBP1 appears to show enhanced affinity for NADH than to NAD+, CtBP has been postulated to be a redox sensor that links the cellular metabolic status to transcriptional regulation.[57] In the Chapter by Goodman, Zhang and colleagues, they discuss the experimental and structural evidences in support of this model. Interestingly, it is possible that there may be a functional relationship between CtBP and the dinucleotide-dependent transcriptional regulator Sir2. The potential relationship between these two transcriptional coregulators has been suggested by a chromatin profiling study in *Drosophila* embryos where 90% of dSir2-recruiting loci were also found to recruit dCtBP.[57a]

Transcriptional Activation

Although CtBPs function predominantly as transcriptional corepressors linking various chromatin-modifying components to DNA-binding repressors, under certain conditions they may function as transcriptional activators. Studies with *CtBP2*-null mouse embryos which exhibit axial truncation phenotypes have revealed that expression of one of the target genes of Wnt3A, *Brachyury*, is lower in E10.5 embryos compared to normal looking E9.5 embryos.[36] This observation suggests that CtBP2 may function as a transcriptional activator of *Brachyury*. A context-specific transcriptional activation function for dCtBP has also been suggested based on transcriptional tethering studies with Gal4-dCtBP in different mammalian cell lines.[58] In contrast to the repression function, it appears that the activation function of CtBP may be indirect. For example, mTcf3, which represses *Brachyury* primarily through the corepressor Gro, contains two divergent CtBP-binding motifs. It is possible that CtBP may activate *Brachyury* by interfering with the repressive function of Gro. It should be noted that a similar antagonism between Gro and CtBP has been observed in the context of transcriptional regulation by Hairy in *Drosophila*.[59]

Cytosolic Functions of CtBP

Role in Membrane Fission and Transport

CtBP1 has also been identified as a 50 kD cytosolic target (designated BARS-50) for ribosylation that is mediated by the fungal toxin brefeldin A (BFA) in the Golgi.[60] The ability of BFA to disassemble Golgi appears to correlate with ribosylation of BARS-50. Protein purification and cDNA cloning identified the rat homolog of CtBP1 as BARS-50[16] (see Chapter by Spano, Hidalgo Carcedo, and Corda). Although the cloned cDNA corresponds to the splice variant CtBP1-S (Fig. 1), it appears that both isoforms of CtBP1 (CtBP1-L and CtBP1-S) may have BARS activity. In vitro studies have revealed that recombinant or purified CtBP/BARS can induce fission of isolated Golgi membrane.[61] During these studies, CtBP1-S was shown to bind acyl-CoA. Spano et al discuss the structural basis for the dual function of CtBP1 (also see Chapter by Lundblad). Based on molecular modeling,[38] they suggest that CtBP1 exhibits fissioning activity when it binds acyl-CoA and assumes an open structural configuration as a monomer and participates in transcriptional regulation when in the dimeric form bound to NAD(H). Although the initial studies ascribed the Golgi membrane fission activity of CtBP/BARS to a slow acyltransferase activity,[61] subsequent studies have suggested that a mutant of CtBP/BARS defective in this activity was able to induce membrane fission with lower efficiency.[62] Studies using mitotic cytosolic extracts from normal rat kidney (NRK) cells that were immuno-depleted for CtBP/BARS and then reconstituted with recombinant *wt* or dominant negative mutants of CtBP/BARS revealed that CtBP is important for the mitotic fragmentation of the Golgi complex.[62] These results have been further extended using living cells that were microinjected with CtBP antibodies or dominant negative mutants or antisense oligonucleotides. The results from such studies have suggested a critical role for CtBP1 in mitotic partitioning of Golgi in the NRK model.

Since fission is a critical step in membrane transport, Luini, Corda and colleagues have also investigated the role of CtBP/BARS in the formation of transport carriers from the Golgi complex to the plasma membrane.[63] Both siRNA-mediated depletion of CtBP1 and the same approaches used to demonstrate a role in mitotic partitioning of Golgi complex were used to demonstrate a role for CtBP1 in dynamin-independent endocytic and exocytic transport pathways in cells of epithelial origin. In contrast to the results on membrane fission and transport obtained with NRK and COS (monkey kidney) cells, studies with mouse embryo fibroblasts (MEF) that are null for both CtBP1 and CtBP2[36] do not appear to show any significant defects in Golgi partitioning. These MEFs also appear to proliferate normally and are not deficient in membrane transport (see Chapter by Hildebrand).[63] Similarly, the *AN* mutants of *A. thaliana* also do not appear to show any Golgi defects (see Chapter by Tsukaya). Spano et al discuss the possibility that 'adaptive' mechanisms during embryonic development might have caused the CtBP-independent Golgi partitioning and transport mechanisms observed in CtBP-null MEF. The cell types could also be a critical determinant for the requirement of CtBP1 for Golgi fission and transport. An interesting question is whether CtBP2 could substitute for CtBP1 in the membrane fission and transport assays. The membrane fission and transport activities mediated by CtBPs may have relevance to central nervous system synapses (see below). This issue is addressed in the Chapter by tom Dieck et al.

CtBPs in Central Nervous System Synapses

The discovery and cloning of RIBEYE (Fig. 1B) as a component of the ribbon synaptic complex revealed a surprising function for CtBPs in central nervous system synapses.[17] Visual and auditory sensory neurons are endowed with the capacity for tonic release of the neurotransmitter. These cells express a synaptic 'ribbon' that tethers clusters of vesicles and transports them to active sites at the plasma membrane (Fig. 3). Although several proteins have been identified in the ribbon complex, RIBEYE appears to be a major constituent.[17] While the B-domain (CtBP2) of RIBEYE is highly conserved between species, the A-domain is divergent, suggesting that the A-domain plays a ribbon-specific structural role in forming the ribbon backbone. Depletion of RIBEYE in zebrafish (by the use of morpholino antisense oligonucleotides) has been shown to result in shorter synaptic ribbons.[20] As discussed in Chapter by tom Dieck et al, Brandstätter and colleagues have discovered that CtBP1 is also a constituent of the ribbon synapses.[19] The role of CtBPs in tethering of vesicles to the ribbon and their mobilization appear to be independent of PXDLS binding.[18] Each ribbon appears to contain ~ 4000 molecules (RIBEYE/CtBPs) that bind to a PXDLS-containing fluorescent peptide probe, thus comprising the majority of the volume (›60%) of the ribbon.[18] Ultra structural studies have revealed that both RIBEYE and CtBP1 colocalize throughout the ribbon structure. The presence of both RIBEYE and CtBP1 may meet the needs of tonic rate release of neurotransmitter. Brandstätter and colleagues have also identified CtBP1 as a constituent of the conventional chemical synapses that do not express RIBEYE.[19] In Chapter by tom Dieck et al the authors propose two roles for CtBPs in chemical synapses, a structural role (backbone of ribbon and ribbon variations) and a role in membrane turnover. They suggest that the lipid binding activity that modulates the curvature of lipid membranes may be important for exocytosis of membrane vesicles. It would be interesting to know if RIBEYE possesses any such activity or facilitates recruitment of CtBP1 (via heterodimerization) to the ribbon synapses. The availability of a knockout mouse model for CtBP1 makes it possible to investigate the role of CtBP1 in central nervous system synapses.

Control of Plant Microtubule Cytoskeleton

As discussed in Chapter by Tsukaya, the *AN* gene of *Arabidopsis thaliana* controls the process of leaf hair (trichome) branching and polarized leaf cell expansion, which influences the leaf shape. An abnormal distribution of microtubules is present in *AN* mutant plants. Genetic

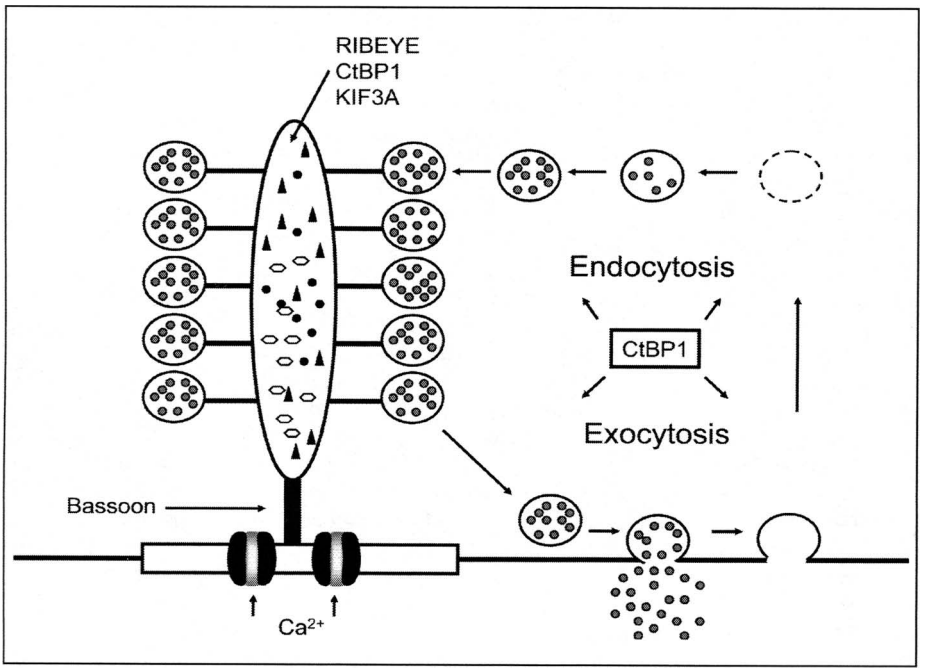

Figure 3. CtBPs in ribbon synapse. The RIBEYE protein is postulated to be the predominant constituent of the ribbon. Other constituents of the ribbon include CtBP1 and the kinesin motor molecule KIF3A.[19] The ribbon is anchored to the presynaptic membrane by the protein Bassoon which is associated with voltage-gated Ca^{2+} channels. CtBP1 is suggested to play a role in membrane turnover during exocytosis and endocytosis of the synaptic vesicles (see Chapter by tom Dieck et al).

studies revealed that the *AN* gene might interact with the gene *ZWICHEL* (*ZWI*),[21] which codes for a protein related to the kinesin motor molecule.[64] The genetic interaction between *AN* and *ZWI* was discovered in double heterozygous plants of certain *zwi* and an allele combinations. Such heterozygous plants contained more trichome branches than the corresponding wild-types. Further, the *zwi* mutants exhibited a phenotype similar to the *an* mutants.[65] Yeast two hybrid analysis also indicated a physical interaction between AN and ZWI proteins. It is interesting to note that the mammalian ribbon synaptic complexes also contain a kinesin related protein KIF3A, in addition to CtBPs.[19] It has been suggested that some AN functions in leaf cell morphogenesis may be linked to directional vesicle trafficking controlled by the microtubule cytoskeleton and motor molecules such as ZWI.[65]

Role of CtBPs in Developmental Processes

CtBP family proteins play critical roles during development of both invertebrates and vertebrates. Homozygous inactivation of the *dCtBP* gene in *Drosophila* is lethal.[12] Embryos with reduced levels of maternal dCtBP exhibit severe segmentation defects,[12,27] which have been attributed to the loss of repression of target genes by several short range transcriptional repressors. The Chapter by Aihara, Perrone and Nibu discusses the activities of short range repressors and dCtBP in the early *Drosophila* embryo. Studies with *Xenopus* embryos have also revealed that CtBPs play critical roles in development by regulating the activities of transcriptional regulators such as Tcf-3, FOG and ZEB-2/SIP1.[24,66-68] In Chapter by Verger, Perdomo and Crossley, they discuss the role of FOG and CtBP in hematopoiesis in *Xenopus* and in *Drosophila.*

In the Chapter by Jeffrey Hildebrand, he discusses his genetic analysis of mice with mutations in the *CtBP1* and *CtBP2* genes.[36] His studies have revealed that the two CtBP isoforms have nonredundant as well as redundant functions during mouse development. *CtBP1*-null mice are viable but are small and less robust. Homozygous inactivation of the *CtBP2* locus results in embryonic lethality between E9 and E10.5, primarily due to defects in placental development. Some of the phenotypes associated with CtBP2-null embryos may be attributed to a reduction in expression of the T-box transcription factor Brachyury. A prominent phenotype associated with the deficiency of CtBP isoforms appears to be the presence of extensive epithelial components in various tissues and organs. This is consistent with the role of CtBP in repressing the expression of various genes important for conferring epithelial phenotype such as E-cadherin.[40] Although the pathways controlled by CtBPs during development remain to be clarified in detail, Hildebrand highlights the link between CtBPs and signaling pathways such as the Wnt and TGF-β/BMP during development. A study with *Drosophila* embryos has revealed a link between dCtBP and modulation of the Wg pathway during development.[69] A more recent analysis of the expression of CtBP1 and CtBP2 genes in avian embryonic development also suggest that the two genes may play functionally redundant roles in development of some tissues and unique roles in development others,[69a] like during mouse embryo development. The Chapter by Hildebrand also highlights the similarities between the phenotypes observed in *CtBP* mutant mice and those of the human syndrome Holoprosencephaly (HPE). Valuable *CtBP* mutant mouse models should facilitate further elucidation of the roles of CtBPs in vertebrate development.

Role in Oncogenesis and Apoptosis

The available evidence suggests that CtBPs may play important roles in tumorigenesis and tumor progression by modulating the activities of oncogenes, signaling pathways, and apoptosis. A role of CtBP in oncogenesis was first inferred from studies with the adenovirus *E1A* oncogene.[1,2,70] Mutations in the C-terminal region of the E1A protein that obliterate the CtBP-binding motif (PLDLS) induced enhanced transformation of primary rodent epithelial cells in cooperation with the activated *Ras* oncogene (Fig. 4). Transformed cells expressing the mutant *E1A* and the *Ras* oncogene were also highly tumorigenic and metastatic. Thus, the interaction of CtBP with the C-terminus of E1A results in suppression of the full oncogenic activity of the *Ras* oncogene. It appears that the hyper-transforming phenotype of E1A C-terminal mutants is specific for cooperative transformation with the *Ras* oncogene, since such E1A mutations are defective in transformation in cooperation with the adenovirus E1B region.[71-73] More recent studies by Grand and coworkers (described in Chapter by Grand et al), also suggest that a mutation within the CtBP-binding motif confers a temperature sensitive phenotype to Ad12 E1A-EIB cooperative transformation. The role of the C-terminal region of E1A in E1A-E1B cooperative transformation may be linked to the inability of E1A C-terminal mutants to induce immortalization of primary cells. The *Ras* oncogene may override an immortalization restriction to induce oncogenic transformation in cooperation with E1A C-terminal mutants. The CtBP-binding motif of E1A is implicated in relief of repression of the telomerase (hTERT) promoter.[74] It is possible that the immortalization defect of C-terminal (exon 2) mutants of E1A may be linked to their inability to activate the hTERT promoter.

Although the mechanism by which CtBP interaction with E1A modulates oncogenic transformation is not fully understood, it appears that most of this activity may be related to relief of CtBP-mediated transcriptional repression by the second exon of E1A. Frisch and coworkers have demonstrated that E1A induced expression of several epithelial genes and E1A mutants defective in interaction with CtBP were partially deficient in activation of these genes.[75] A microarray analysis, which compared the gene expression profiles of cells expressing *wt* E1A or an E1A mutant lacking the CtBP-binding motif, identified a number of genes that were activated by *wt* E1A and not by the C-terminal mutant.[76] These genes included those involved in tumor progression and growth suppression.[76] A different gene expression profiling study

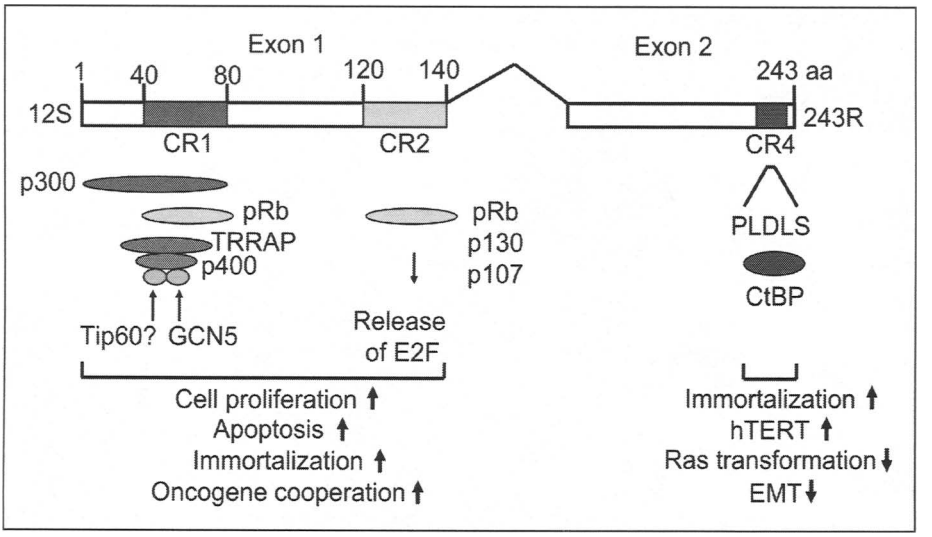

Figure 4. Activities of adenovirus E1A oncoprotein. The *E1A* gene of adenovirus codes for two major protein isoforms (243R and 289R) which are identical except for the presence of a 46 amino acid domain (CR3) that is unique to 289R. The activities of the 243R protein are shown in the figure. The N-terminal half (exon 1) interacts with multiple cellular growth regulatory molecules (p300, TRRAP-p400 complex and pRb family proteins) and contributes to cell proliferation and oncogene cooperation. The C-terminal region (exon 2) interacts with CtBP through the PLDLS motif located within the conserved CR4 region. E1A interaction with CtBP results in suppression of *Ras* oncogene cooperation and EMT by relieving repression of epithelial genes such as E-cadherin.

using CtBP-null mouse embryo fibroblasts (MEF) and CtBP1-rescued MEF has revealed that several epithelial (such as cytokeratins, tight junction components and lamins) and pro-apoptotic (such as *PERP*, *Noxa* and *Bax*) genes are activated in the absence of CtBP[40] (see Chapter by Frisch). Thus, it appears that the enhanced transforming properties of *E1A* mutants may be related their inability to relieve CtBP-mediated repression. The activated *Ras* oncogene is known to induce epithelial to mesenchymal transition (EMT) with loss of membranous E-cadherin expression.[77] The hypertransforming (in cooperation with oncogenic *Ras*) mutants of *E1A* may permit unimpeded propagation of *Ras* activity in modulating EMT.

The above-mentioned studies that suggest the C-terminal region of E1A may serve as a tool to inactivate the transcriptional functions of CtBPs. It is possible that the second exon of *E1A* that includes the PLDLS motif could be exploited as a therapeutic agent for certain malignancies in which the expression of tumor-restraining genes is repressed by CtBP-dependent repressors. A cellular protein, Pinin/DRS (*Pnn*), implicated in mRNA processing has also been reported to relieve CtBP-mediated repression of E-cadherin.[78] It is possible that *Pnn* may modulate oncogenesis by regulating EMT in a fashion analogous to E1A. It appears that certain apoptotic stimuli may also mimic the effect of proteins such as E1A and Pnn in neutralizing CtBP functions. Goodman, Zhang and coworkers have shown that in response to exposure to UV, the homeodomain-interacting protein kinase (HIPK2) phosphorylates CtBP1 at Ser-422 resulting in rapid ubiquitination and degradation of CtBP1,[79,80] which is accompanied by apoptosis. It would be interesting to know if phosphorylation-mediated clearance of CtBP-1 also results in activation of the various pro-apoptotic genes (i.e., *PERP*, *Noxa*, and *Bax*) that are activated in CtBP knockout cells.

Among cellular oncogenes, the activity of *Evi-1* is modulated by direct interaction with CtBP. The expression of the *Evi-1* oncogene is activated in human myeloid leukemia and

myelodysplastic syndromes.[81] It is also expressed as a t(3;21) fusion product with AML-1 in chronic myelocytic leukemia.[82] Evi-1 inhibits Smad-activated transcription of TGF-β/activin/BMP (bone morphogenetic protein)-responsive genes by recruiting CtBP.[83-86] In Chapter by, Verger, Perdomo, and Crossley, the authors discuss the role of CtBP in conjunction with the *Evi-1* gene, the *AML/Evi-1* fusion gene , the more recently discovered *AML1/FOG-2* fusion gene and the *MLL* (mixed lineage leukemia) gene in leukemogenesis. A direct interaction of CtBP with the viral oncogenes EBNA3A and EBNA3C is also required for the immortalization and cooperative transformation activities of the EBV oncogenes.[87,88]

Gene expression studies in CtBP-null cells and certain protein interaction studies raise the possibility that there may be some cross-talk between the p53 and CtBP pathways in modulating oncogenesis. The pro-apoptotic genes, *PERP, Noxa,* and *Bax* as well as p21 are well known target genes for p53. The expression of these genes was shown to be highly activated in CtBP-null MEFs.[40] However, reporter-based assays (using p53-responsive promoter constructs) performed in the MEFs have suggested that CtBPs may not directly antagonize the activity of p53. A different protein interaction study identified interaction between Hdm2 and CtBP2.[89] The Hdm2/Mdm2 oncoprotein is known to mediate its oncogenic activity by inactivating p53 through multiple mechanisms and is also known to possess an intrinsic transcriptional repressor activity.[90] Based on these results, it has been suggested that CtBP2 may cause promoter-selective inhibition of transcription of p53-responsive genes through interaction with Hdm2/Mdm2.[89] The potential link between the p53 pathways and CtBP pathways merit further investigation.

In addition to the potential tumor-promoting activities of CtBP by regulating the activities of oncogenes and tumor suppressor genes, the available evidence also suggests that CtBP has a role in tumor suppression in the colon. During a search for *Drosophila* proteins that complex with the E-APC (adenomatous polyposis coli) protein, Hamada and Bienz identified dCtBP as an APC-interacting protein.[69] They extended these results to the human APC, an important tumor suppressor in the colon. Hamada and Bienz have demonstrated that CtBP binds directly to APC through PXDLS-like motifs conserved between the fly and mammalian APC proteins. The interaction between APC and CtBP results in sequestration of the APC/β-catenin

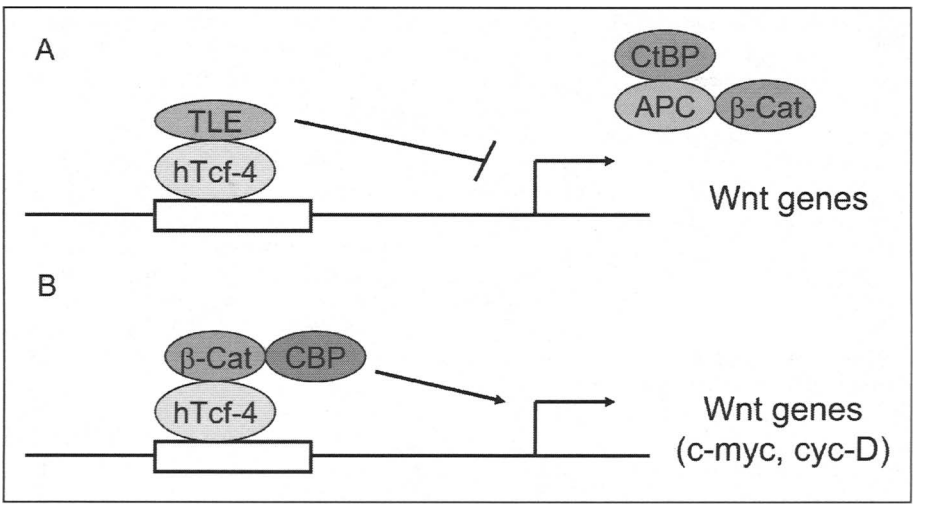

Figure 5. Modulation of Wnt gene expression by CtBP. The model proposed by Hamada and Bienz[69] predicts that direct interaction of CtBP with the APC protein antagonizes Tcf-4-mediated transcription of Wnt target genes in the colon by sequestering free nuclear β-catenin. In addition to the deprivation of nuclear β-catenin, the transcriptional activity of Tcf-4 may be compromised by binding of the corepressor TLE (Gro). A. Inhibition of Wnt genes in normal colon cells. B. Activation of Wnt genes in colon cancer cells.

complex, thereby redirecting free nuclear β-catenin away from the Wnt transcription factor hTcf-4 (Fig. 5). Functional cooperation between CtBP and APC is consistent with the model that CtBP may serve a tumor suppressor role in the colon. The observation that some colon cancer cell lines (e.g., COLO320) express APC truncations lacking the CtBP-binding sequences lends support to this view.

Concluding Remarks

In the last ten years since the cloning of the founding member of the CtBP family proteins, these proteins have evolved from an enigmatic state to a state of much biological importance. A number of critical studies, particularly with dCtBP have been instrumental in establishing a clear role for CtBP family members in regulating transcription. Similarly, the characterization of the CtBP super complex from human cells has been a significant advancement towards understanding the mechanisms of transcriptional repression in mammalian cells. Since the mammalian CtBP1 protein complex appears to contain unique constituents not present in the dCtBP complex, future studies will illuminate a common mechanism shared by the invertebrate and vertebrate CtBPs. Future studies are needed to establish a clear transcriptional role for the plant homolog, ANGUSTIFOLIA. Although CtBPs were the first transcriptional regulators identified to also contain a metabolic enzyme fold, recently such enzymatic constituents have been identified in various transcription complexes. Since there is an absolute conservation of the D2-HDH fold among the animal CtBPs, the next challenge would be to identify the relevant substrate(s) for these enzymes. Detailed investigation into the potential regulation by the NAD(H) dinucleotides of the transcriptional activities of CtBP in tumor cells would be important to gain insight into designing strategies for anti-cancer therapeutic intervention and to discover potential cross-talks between pathways controlled by other dinucleotide-regulated transcriptional regulators such as Sir2. The studies on membrane associated functions of CtBP1 have been instrumental in unraveling the dual activity of CtBP1. Compelling evidence that CtBPs also function as synaptic proteins warrants additional investigations on the role of CtBPs in membrane turnover. Apart from the functional importance of the CtBP family proteins, the genomic organization of these genes have illuminated novel strategies employed by vertebrates to encode proteins (e.g., RIBEYE and CtBP2) of diverse functions within a single gene locus to achieve genome compaction. The roles of CtBP in modulating oncogenic outcomes via EMT and apoptosis raise a promising possibility that CtBPs may be good anti-neoplastic drug targets.

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CHAPTER 2

Transcriptional Repression by the CtBP Corepressor in *Drosophila*

Hitoshi Aihara, Lorena Perrone and Yutaka Nibu*

Abstract

Transcriptional repression is essential for patterning gene expression in the early *Drosophila* embryo. Biochemical and genetic studies on *Drosophila* C-terminal binding protein (dCtBP) have provided solid evidence that dCtBP acts as a corepressor for several transcriptional repressors. Similarly to mammalian CtBPs, dCtBP interacts with a short peptide motif, PxDLS, or related motifs. It appears that dCtBP is essential for short-range transcriptional repression in the early embryo. In contrast, it has been recently reported that dCtBP participates in Polycomb-mediated long-range repression. In this chapter, we will review how the dCtBP corepressor functions, from the biochemical, developmental, and genetic point of views.

Introduction

Numerous biochemical and genetic analyses have established *Drosophila melanogaster* (fruit fly) as one of the most accessible model systems for studying transcriptional networks, regulatory elements and factors controlling them. During early *Drosophila* embryogenesis, a hierarchy of gene networks consisting of maternal and zygotic genes (gap, pair rule, segmentation polarity genes, etc.) progressively divides the embryo into increasingly precise segments/territories.[1,2] This patterning process further depends on broadly distributed activators and localized sequence-specific repressors to refine the initial segmentation boundaries.

In 1995, Chinnadurai and colleagues cloned the human *CtBP1* (*hCtBP1*) gene. hCtBP1 interacts with the adenovirus E1A oncoprotein through a specific amino acid motif, PLDLSCK.[3] In 1998, using yeast two-hybrid screens, two laboratories identified dCtBP as a factor that physically interacted with three transcriptional repressors involved in embryonic patterning: Knirps, Snail, and Hairy.[4,5]

Structure of the *dCtBP* Gene and Its Proteins

Drosophila carries a single copy of the *dCtBP* gene on the right arm of the third chromosome (located cytologically at 87D8-87D9),[4-6] whereas human and mouse have two highly related CtBP genes, CtBP1 and CtBP2.[7,8]

The annotation of the *dCtBP* gene, based on the analyses of both *dCtBP* expressed sequence tag (EST) clones and the fly genome sequence, predicts 386 amino acids (aa) and four splicing variants differing in 5' untranslated region of the mRNA (Fig. 1A).[6] Due to heterogeneity of the 5' termini, *dCtBP* was predicted to be transcribed by four separate promoters. In protein

*Corresponding Author: Yutaka Nibu—Department of Cell and Developmental Biology, Weill Medical College of Cornell University, 1300 York Avenue, Box 60, A308, New York, New York 10021, U.S.A. Email: yun2001@med.cornell.edu

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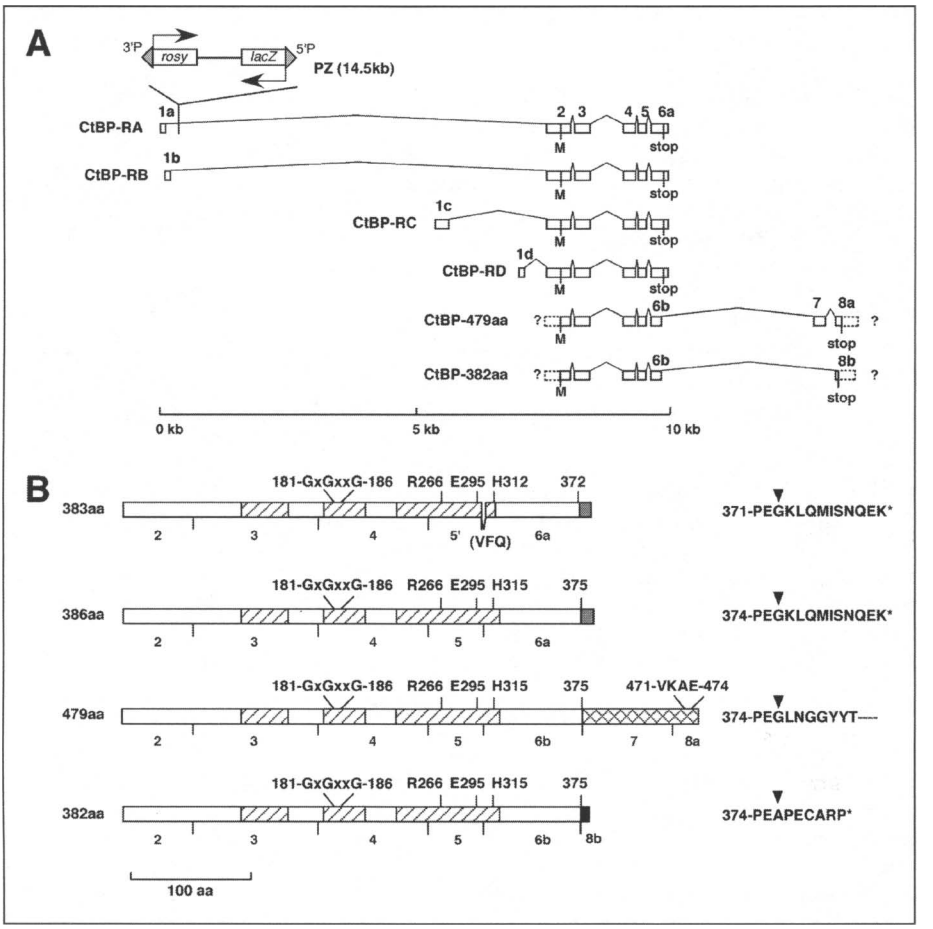

Figure 1. Structure of the *dCtBP* gene and the dCtBP protein isoforms. A) Organization of the *dCtBP* transcription unit. Each exon is shown as an open box and assigned a number on top. Introns are indicated by thin lines. Locations of the translation initiation site and the stop codon are indicated by "M" and "stop", respectively. Four transcripts, CtBP-RA to -RD, are annotated in the fly base and all encode the 386aa isoform.[4,5] CtBP-479aa and CtBP-382aa are also shown in this panel based on the previous studies and our unpublished results, but the exon/intron structures 5' of the translation initiation and 3' of the stop codon are unknown. The insertion of the PZ P-element (14.5 kb) located 500 bp downstream of exon 1a in the *dCtBP*^{03463} mutant disrupts dCtBP function. B) Schematic structure of dCtBP proteins. The NAD^+/NADH binding motif (GxGxxG) and the catalytic triad (arginine, glutamic acid, and histidine) are conserved among all the dCtBP isoforms. Hatched boxes indicate regions of high similarity with the dehydrogenases. Numbers and thin lines below each rectangle indicate exons. Shaded, double hatched, and solid boxes at the C-termini represent portions of splicing variants. The amino acid sequences around the alternative splicing points are shown right to the panel. Asterisks indicate the C-terminus ends. The splicing points (arrowheads) are G376 in dCtBP 479aa or A376 in the 382aa isoform. The 383aa isoforms derive from a shorter exon 5, lacking three amino acids, VFQ. "VKAE" in the 479aa isoform is a similar motif to the sumoylation site.

coding regions, at least four alternatively spliced forms obtained from the yeast two-hybrid screens, 382aa, 383aa (accession number AB011840), 386aa (accession number AJ224690), and 479aa, have been reported (Fig. 1B).[4,5,9,10] The CtBP family proteins are similar to

NAD^+-dependent D-isomer-specific 2-hydroxy acid dehydrogenases.[7,8] The NAD^+/NADH binding motif (GxGxxG) and the catalytic triad (arginine, glutamic acid, and histidine residues) are conserved among all isoforms (Fig. 1B).[4,10] hCtBP1 has been demonstrated to be a functional dehydrogenase, but it is still unclear whether the same is true for dCtBP.[11-13]

Northern blot analyses have shown that three major transcripts, 2.5, 2.7, and 4.0kb long, are expressed during all stages of *Drosophila* development.[5] A 3.5kb transcript is abundant in adult females and embryos. So far, it is not known which mRNAs correspond to each alternatively spliced form. Maternally expressed *dCtBP* is uniformly distributed throughout the early embryo.[4,5] dCtBP is primarily detected in nuclei by antibody staining in the early embryo, although its sequence does not include an apparent nuclear localization signal.[4]

Factors Interacting with dCtBP

So far, 12 *Drosophila* factors have been shown to interact with dCtBP or the vertebrate CtBPs (Fig. 2). CtBP is known to specifically interact with a specific five-residue motif, PxDLS.[7,8] The Snail, Knirps, Krüppel, Brinker, Teashirt, and Hairless proteins contain the PxDLS motifs that are recognized by dCtBP in vitro.[4,9,14-16] U-shaped, a regulator of embryonic hematopoiesis, interacts with dCtBP in vitro and the PxDLS motif of its vertebrate homolog, Fog, is recognized by CtBP.[17] In coimmunoprecipitation assays, Zfh-1, which regulates somatic and cardiac myogenesis, interacts with both hCtBP1 and mCtBP2, and the PxDLS motifs of its vertebrate homolog ZEB are essential for CtBP binding.[18] dCtBP can also bind motifs other than PxDLS. For example, the DNA-binding factor Tramtrack69, that regulates eye development, has the PPDLS motif at the C-terminus, but dCtBP binds its N-terminus including the BTB domain rather than the PxDLS motif.[19] In addition, Hairy, E(spl)m delta, and E-APC have related sequences that bind dCtBP in vitro.[5,20]

Short-Range Transcriptional Repression and dCtBP in the Early Embryo

Reduction of maternal dCtBP protein causes severe patterning defects in both the anterior-posterior and dorsal-ventral axes of the early embryo.[5,9] The expression of several marker genes is severely altered in the $dCtBP^{03463}$ mutant embryo, since the activities of major repressors, Krüppel, Knirps, and Snail, are impaired.[9,21-23] Transgenic assays have shown that, in *dCtBP* mutants, these repressors cannot function, suggesting that dCtBP is required to mediate the transcriptional repression of these different transcription factors.[9,21] These repressors carry a sequence similar to the PxDLS motif, PxDLSxR/K/H, that interacts with dCtBP in vitro.[4,9] Mutant forms of these repressors, lacking the PxDLS motifs, fail to repress their target genes in transgenic embryos.[4,9,24] Thus, the PxDLS motif is essential for dCtBP binding as well as Krüppel-, Knirps-, and Snail-mediated repression.

These repressors are called short-range repressors as they work over distances of less than 100 bp to inhibit adjacent activators.[9,25] Short-range repression is unique in that it allows enhancers to work independently of one another. Giant (bZip), another short-range repressor, is partially dependent on dCtBP.[26,27]

The gap genes, Krüppel, Knirps, and Giant, regulate expression of primary pair-rule genes, for example, the *even-skipped* (*eve*) gene (Fig. 3A). The *eve* gene harbors five blastoderm enhancers, located 5' and 3' of the transcription unit, that control one or two stripes.[28-30] These enhancers are typically 300 bp to 1 kb in length and contain clustered binding sites for activators and repressors.[31] The borders of individual stripes are formed by localized short-range repressors which turn off transcription via a concentration threshold mechanism.[32] For example, the maternal Bicoid (homeobox) gradient along the anterior-posterior axis activates the *eve* stripe 2 enhancer in a broad anterior domain, but the Giant and Krüppel repressors restrict the pattern within sharp stripe borders (Fig. 3B).[28,33] Krüppel competes with Bicoid for common sites in a dCtBP-independent manner (Fig. 3C).[21,28] In addition, when positioned within 100 bp from Bicoid sites, Krüppel mediates repression via a quenching mechanism

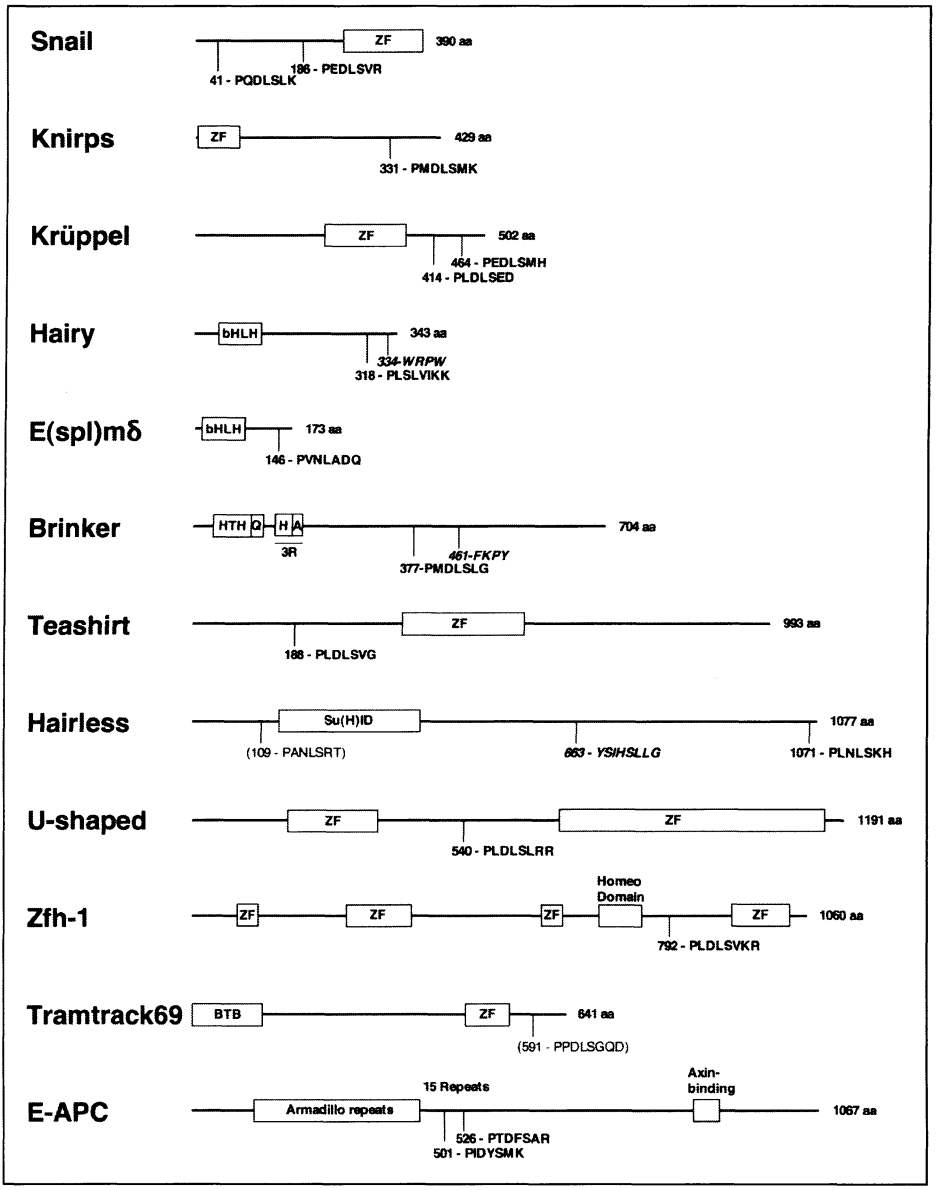

Figure 2. Summary of the structure of dCtBP interacting factors. dCtBP interacting motifs are indicated in bold. Amino acid sequences similar to the dCtBP interaction motif are indicated by brackets. Groucho interacting motifs are shown in italic. ZF: zinc finger, bHLH: basic helix-loop-helix, BTB: broad complex Tramtrack bric-a-brac/Pox virus and zinc finger, Su(H) ID: Su(H)-interaction domain, Q: poly-glutamine region, H: histidine rich region, A: poly-alanine region.

through the corepressor dCtBP (Fig. 3D).[9] Even though expression of *Krüppel* and *eve* stripe 3 overlap, the binding of Krüppel to the stripe 2 enhancer does not interfere with stripe 3 expression due to the limited range of action of this repression (Fig. 3A,B).

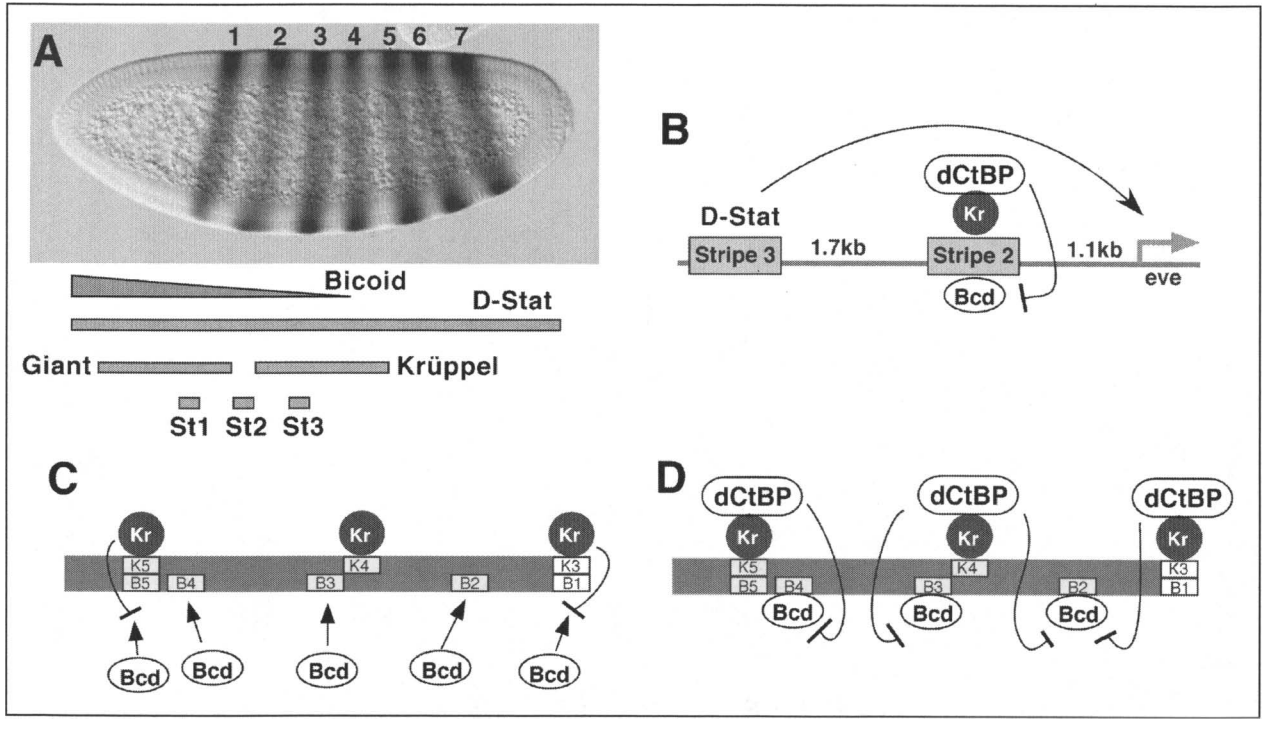

Figure 3. The short-range Krüppel repressor is required for the regulation of *eve* expression. A) *eve* expression in an embryo 3-hr after fertilization. The embryo is oriented with anterior to the left and dorsal up. *eve* expression is detected by in situ hybridization with a digoxigenin-labeled *eve* antisense RNA probe. The number of each stripe is indicated above the embryo. The gray boxes represent spatial expression patterns of the indicated factors and each stripe (St). B) The 480-bp *eve* stripe 2 and the 500-bp stripe 3, which are separated by 1.7kb, are located in the 5'-flanking region of the *eve* locus. In cells expressing stripe 3, the Krüppel-dCtBP complex inhibits Bicoid (Bcd) bound to the stripe 2 enhancer, without interfering with the D-Stat activators bound to the stripe 3 enhancer. C) The stripe 2 enhancer contains five Bicoid binding sites (B1 to B5) and three Krüppel binding sites (K3 to K5). Krüppel probably prevents Bicoid from binding to B1 and B5 via the dCtBP-independent competition mechanism. D) The Krüppel-dCtBP complex represses Bicoid bound to B2, B3, and B4 sites via the dCtBP-dependent quenching mechanism.

Along the dorsal-ventral axis, the maternal Dorsal (rel domain) nuclear gradient activates several genes including *rhomboid, short gastrulation, singleminded, ventral nervous system defective* and others, in both ventral and lateral regions of early embryos, but the Snail repressor, that interacts with dCtBP, continues to keep these genes off in the ventral mesoderm.[9,34,35]

When fused to the Gal4 DNA-binding domain, dCtBP (383aa and 479aa) represses expression of the *lacZ* reporter through UAS binding sites in transgenic embryos in a manner indicative of short-range repression.[9,10] Disruption of the NAD^+/NADH binding motif of dCtBP (479aa) abolishes repression activity in transgenic embryos, while a mutation of the catalytic histidine does not abrogate repression activity.[10] However, how dCtBP mediates short-range repression is still unclear.

Histone-Modifying Enzymes and dCtBP

It is known that the mechanisms of repression by mammalian CtBP are HDAC-dependent or -independent as well as histone methyltransferase (HMT)-dependent.[7,8,13] It is, however, still unclear whether dCtBP can associate with HDACs and HMTs, except for the case of dCtBP in PcG-mediated repression (see below). First, the short-range repressors function perfectly in *dRpd3* (*dHDAC1*) mutant embryos, that exhibit segmentation defects due to the loss of Eve repressor activity.[36] Second, in reporter assays using *Drosophila* S2 cells, the repression activities of Knirps, Giant, and dCtBP are insensitive to the HDAC inhibitor trichostatin A (TSA), while Groucho/dRpd3-mediated repression is TSA sensitive.[37] Third, HDAC activity is not detected in immunoprecipitates containing Myc-tagged dCtBP derived from *Drosophila* S2 cells.[38] Fourth, the zinc finger Hunchback repressor participates in PcG repression involving HMT and HDAC.[39,40] Hunchback represses the outer borders of *eve* stripe 3/7 and 4/6 in a concentration-dependent manner.[30,32] However, Hunchback can function without dCtBP in the early embryo.[22,23]

dCtBP Interference with Hairy-Bound Groucho

In the early embryo, Hairy, a bHLH DNA-binding repressor, establishes segments as a primary pair-rule gene by repressing *fushi tarazu* (*ftz*) expression.[41] Hairy represses *huckebein* expression and is essential for controlling both the cell size and cell shape of the embryonic salivary gland lumen.[42] Hairy also represses the proneural gene *achaete* during development of the larval peripheral nervous system (PNS).[43]

Hairy interacts with Groucho and mediates long-range repression (Fig. 2).[24,44,45] dCtBP weakly recognizes a PLSLVIKK motif in the Hairy protein.[5,45] However, it appears that dCtBP inhibits the repression activity of Groucho/Hairy rather than act as a corepressor.[45] This model is supported by the following evidence. Hairy continues to repress reporter expression in the absence of dCtBP.[9] Segmentation defects and altered *ftz* expression in *hairy* mutants are suppressed by removing one copy of *dCtBP*, but the loss of one dose of *Groucho* enhances the *hairy* phenotype.[5,38] When Hairy is misexpressed in the anterior regions of embryos, several potential Hairy target genes are repressed.[45] Mutations in the Groucho interaction motif (WRPW) attenuate the repression activity, while disruption of the weak dCtBP interaction motif (PLSLVIKK) increases it.[45]

The genome-wide distributions of Hairy, Groucho, and dCtBP have been determined by the chromatin profiling DamID method.[46] In *Drosophila* Kc cell lines, Hairy, Groucho, and dCtBP are bound to 40, 155, and 496 sites, respectively. Most of the Hairy targets (38 out of 40 sites) recruit dCtBP, but not Groucho. Intriguingly, only one site is recognized by both Hairy and Groucho, while dCtBP and Groucho colocalize at only one site. dCtBP binds to all of the 34 targets recognized by both Hairy and another Hairy interacting factor dSir2 (Histone deacetylase).[46,47] In addition, most of the dSir2 targets (97 out of 107 sites) recruit dCtBP.[46] dCtBP and dSir2 are both NAD^+-dependent factors. Although dSir2 does not directly associate with dCtBP in vitro,[47] a combination of dCtBP and dSir2, responding to the redox state of the cells, may modulate transcriptional activities of these transcriptional corepressor proteins.

Multiple Repression Domains

Some of the dCtBP-interacting factors, such as Krüppel and Knirps, possess multiple repression domains. Unlike Hairy, these domains can function qualitatively and/or quantitatively different, or act in a tissue/cell-type specific manner.

Krüppel has two evolutionarily conserved repression domains.[48] The C-terminal repression domain (402-502aa), that is dCtBP-dependent, is functional in both, tissue culture cells and in transgenic embryos.[9,21,48,49] The N-terminal repression domain (62-92aa) is active in tissue culture cells, but is dispensable in blastoderm embryos, suggesting a cell-type specific effect.[21,48,50]

Knirps has two repression domains, dCtBP-dependent and -independent domains, that function in both transgenic embryos and in *Drosophila* S2 cells.[22,23] In *dCtBP* mutant embryos, expression of *eve* stripes 4/6 is derepressed, but Knirps-mediated repression of the stripes 3/7 is not affected. However, the mutant form of Knirps lacking the dCtBP-dependent repression domain is able to repress not only stripes 3/7 but also stripes 4/6 in a concentration dependent manner. Taken together, these results indicate that the two domains contribute to the full repression activity of Knirps quantitatively rather than qualitatively.

Role of dCtBP in Signaling Pathways

dCtBP participates in three major signaling pathways, Notch, Wingless, and Dpp. In the presence of Notch signaling, the DNA-binding factor Su(H) acts as an activator.[15] In contrast, binding of Hairless converts Su(H) into a repressor in unstimulated cells. Hairless interacts with both Groucho and dCtBP through the YSIxxLLG and PLNLSK motifs, respectively. During development of the *Drosophila* adult mechanosensory bristle, both corepressors contribute to the function of Hairless.

The Wingless (Wg) ligand stimulates downstream gene expression through a DNA-binding factor dTCF/Pangolin and a coactivator Armadillo (beta-Catenin).[51] In cells lacking a Wg signal, however, dTCF keeps its target genes off by recruiting Groucho.[52] Recently, *Drosophila* E-APC, which interacts with Armadillo, has been identified as a dCtBP-interacting factor.[20] dCtBP specifically recognizes conserved 15aa repeats of E-APC in vitro, but it does not bind Armadillo. Genetic assays in eyes and wings show that dCtBP antagonizes Armadillo and hence, Wg signaling is repressed at two levels by Groucho and dCtBP. The association of CtBP and APC is also conserved in mammalian cells, and the CtBP-APC interaction reduces TCF-mediated transcription, suggesting that CtBP may serve as a tumor suppressor in the colon.

Some genes activated by Dpp signaling are repressed by Brinker, a helix-turn-helix transcriptional repressor.[16,53] Brinker has at least three repression domains, a dCtBP interacting domain, a Groucho interacting domain, and a newly identified region (3R).[53] In the early embryo, *zerknüllt* is activated by the Dpp signal transducer p-Mad, but Brinker competes for binding to the Mad binding sites thus determining the spatial limits of *zerknüllt* expression.[54] In contrast, repression of *pannier* and *tolloid* by Brinker is Groucho-dependent.[16,55] During the development of the fly wing, 3R is sufficient to repress *optomotor-blind*, while interaction of Brinker with either Groucho or dCtBP is required for repression of *spalt* and *brinker* itself.[16,53]

In the developing *Drosophila* midgut, the dTCF-mediated activation of the Hox gene *Ultrabithorax* (*Ubx*) is repressed by the nearby-bound Brinker that interacts with both Teashirt and dCtBP.[56] Teashirt, interacting with dCtBP, also represses *modulo* expression in the T1 segment of the embryonic epidermis.[14]

Polycomb Group Proteins and dCtBP

Vertebrate CtBPs may play a role in repression by Polycomb group (PcG) proteins.[57,58] PcG proteins maintain Hox genes in a stably and heritably silenced state during development in *Drosophila* and vertebrates.[59] *Drosophila* PcG proteins consist of up to 15 genes and can fall into two classes; the 2-6 MDa Polycomb repressive complex 1 (PRC1) and the 400-600 kDa PRC2 complex.[59] PRC2 is recruited to Polycomb response elements (PRE), located far from the promoter, by the DNA-binding factor Pleiohomeotic. Histone H3 is epigenetically marked

first, by the deacetylation through dRPD3 which interacts with Enhancer of zeste [E(z)], HMT, in the PRC2 complex, and subsequently by the methylation through E(z) which preferentially methylates lysine 27 of histone H3.[60-62] The methylation of histone H3 defines the landing zone for the PRC1 complex to PREs, since the methylated histone H3 is bound by Pc in the PRC1 complex. Then the PcG proteins spread along the chromatin over a few thousand bases to prevent access of activators to their binding sites.[63,64] Alternatively, PcG proteins bound to PREs interact with the TFIID complex formed on the core promoter via a looping mechanism, to interfere with transcriptional initation and hence block transcription.[65,66]

Mammalian YY1, a Pleiohomeotic homolog, mediates repression in a PcG-dependent fashion involving dCtBP in transgenic *Drosophila* embryos.[67] When fused to the Gal4 DNA-binding domain (Gal4-YY1), YY1 behaves as a PcG protein in transgenic embryos. Both the repression activity of Gal4-YY1 and Gal4-Pc are completely lost in heterozygous *dCtBP*^{03463} mutant embryos. Chip assays and larval polytene chromosomes staining assays suggest that removal of one copy of *dCtBP* clearly attenuates DNA binding of Gal4-YY1 as well as recruitement of endogenous Pc.[68] Thus, dCtBP apparently plays a role in PcG-mediated repression by controlling DNA binding of YY1 and recruitement of PcG. In contrast, Krüppel is still able to bind DNA in the absence of dCtBP.[21]

The PcG can function over distances of more than 1kb to silence transcription and this mechanism is different from dCtBP-mediated short-range repression. Human PC2, a homolog of *Drosophila* Pc, has turned out to be a SUMO E3 ligase and actually CtBP1, unlike CtBP2, is sumoylated.[58,69] One of the dCtBP isoforms, 479aa, contains a putative sumoylation motif, VKAE, (Fig. 1B). Hence, it is unresolved whether sumoylation of dCtBP can change its range of action in vivo.

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CHAPTER 3

CtBP and Hematopoietic Transcriptional Regulators

Alexis Verger,* Jose Perdomo and Merlin Crossley

Abstract

The C-terminal binding proteins (CtBPs) are ubiquitous corepressors that recruit histone-modifying enzymes to a variety of sequence specific DNA-binding proteins and other transcriptional regulators. CtBPs appear to play an important role in mediating repression and transforming activities of a variety of hematopoietic transcription factors such as Basic Krüppel-like Factor/Krüppel-like Factor 3 (BKLF/KLF3), Friend of GATA (FOG), Evi-1 and members of the Ikaros family. Mice lacking CtBPs die during embryonic development and exhibit defects in a wide range of developmental processes, including aberrant heart formation and absence of blood vessels in the yolk sac. The ongoing identification of repressed target genes and interacting transcriptional partners will help to unravel the contributions of CtBP proteins to hematopoiesis.

Introduction

Hematopoiesis is the process through which the various blood lineages (erythrocytic, lymphocytic, monocytic/myelocytic, granulocytic and thrombocytic) develop from self-renewing, pluripotent stem cells.[1] This process is tightly regulated by the action of growth factors that signal to lineage restricted or widely expressed transcription factors and their associated coregulators (Figs. 1, 2). These factors then orchestrate lineage commitment by activating and repressing defined sets of target genes. For example, the zinc finger protein GATA-1 and its cofactor FOG are involved in coordinating the expression of genes that drive erythrocytic and megakaryocytic development.[2,3]

Understanding the transcriptional networks that coordinate such programs of gene expression is an important focus in the study of cell differentiation. Accumulating evidence suggests that the corepressor C-terminal binding protein (CtBP) is an important regulator of hematopoietic homeostasis by virtue of its physical interaction with hematopoietic transcription factors such as BKLF, Evi-1, FOG and Ikaros. This chapter addresses the mechanisms of transcriptional repression and the role of CtBP in development, hematopoiesis and leukemogenesis.

CtBP Proteins during Development

CtBP1 is the founding member of the CtBP family of corepressors. It was first identified as an E1A interacting protein that negatively modulates the oncogenic transformation activity of E1A.[4] Subsequently, highly homologous human and mouse proteins termed CtBP2 were

*Corresponding Author: Alexis Verger—School of Molecular and Microbial Biosciences, University of Sydney, 2006 New South Wales, Australia. Email: a.verger@mmb.usyd.edu.au

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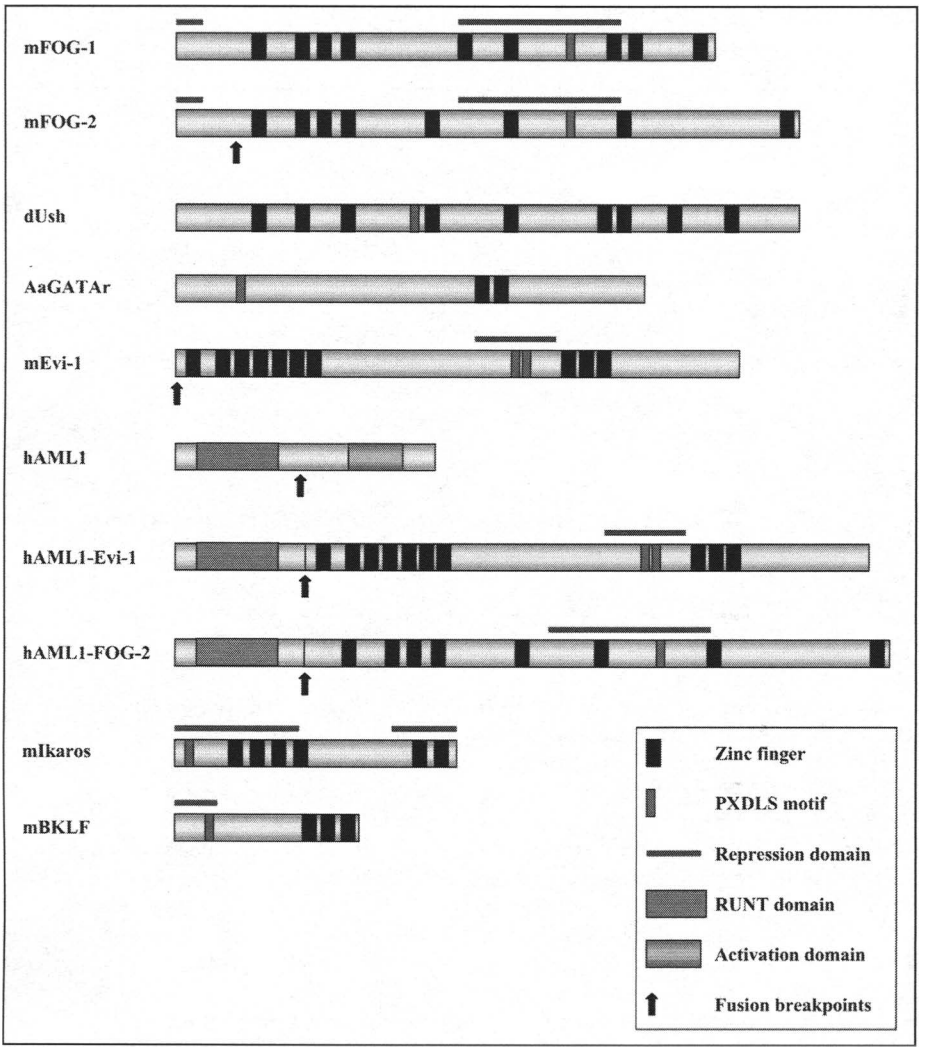

Figure 1. Schematic representation of CtBP partners discussed in this chapter. Structures of mFOG-1, mFOG-2, dU-shaped, AaGATAr, mEvi-1, hAML1, hAML1/Evi-1, hAML1/FOG-2, mIkaros and mBKLF are shown. Zinc fingers are represented as vertical solid bars, the PXDLS CtBP binding motif is indicated as red vertical bars. Blue overlining indicates the repression domains containing the CtBP binding motif. A color version of this figure can be viewed at http://www.Eurekah.com.

identified by analysis of EST data bank sequences and in a yeast two-hybrid screen against the erythroid transcription factor BKLF.[5,6]

CtBP1 and CtBP2 are widely expressed and are often coexpressed. Knockout studies have revealed that *CtBP1*-null mice are viable while *CtBP2*-null embryos die by E10.5.[7] Thus the functions of CtBP2 cannot be assumed by CtBP1. Further evidence for distinct functions comes from an examination of expression patterns. CtBP1 is expressed in the thymus and peripheral blood leukocytes, whereas CtBP2 is not readily detected.[8,9] In human cancer lines, differences in expression are common with high expression of CtBP1 in chronic myelogenous leukemia K-562 and lymphoblastic leukemia MOLT-4 cell lines.[9] Conversely, CtBP2 is readily

Table 1. Hematopoietic genes up-regulated in CtBP -/- cells

Gene	Function
Ferritin light chain 1	Iron storage protein
TGFβ3	Negative regulator of hematopoiesis
Pre B-cell leukemia transcription factor 1 (PBX)	Homeodomain –containing Hox co-factor, fused to E2A in acute lymphoblastic leukemia.
Hemoglobin alpha, adult chain 1	erythrocytes
B-cell receptor-associated protein 31 (BAP31)	Endoplasmic reticulum membrane-sorting protein of lymphocytes
Zinc finger protein multitype 1 (FOG-1)	Essential co-factor of GATA-1 during erythroid and megakaryocyte differentiation
Krüppel like factor 3 (KLF3/BKLF)	Transcriptional repressor expressed in erythroid cells
Erythrocyte protein band 7.2	Red cell membranes
CD4 antigen	T cells
B-cell receptor-associated protein 37 (BAP37)	Prohibitin, an inhibitor of cell proliferation
Zeta-chain (TCR) associated protein kinase (ZAP 70)	TCR-mediated signal transduction
Ankyrin 1	Erythroid specific

Hematopoietic CtBP target genes extracted from reference 12. See text for details.

detected in the forming placenta while CtBP1 is not. *CtBP2*-null embryos are devoid of blood vessels suggesting that CtBP2 has a specific and essential role in angiogenesis.[7]

While the two proteins are clearly non redundant, there is evidence that they do have overlapping functions. The phenotypes of compound heterozygotes with various combinations of *CtBP1* and *CtBP2* alleles provided strong evidence for this.[7] Overall, the myriad developmental defects seen in *CtBP* mutant embryos (axial truncations, delayed neural development, defects in heart morphogenesis) are consistent with the wide diversity of CtBP1 and CtBP2's many interacting partners[10,11] (see chapter by Hildebrand of this book for more details).

Cells derived from mutant mouse embryos have been used to address more precisely the functions of CtBP proteins and identify their relevant target genes.[12] Microarray analysis of CtBPs-knockout versus CtBP-rescued mouse embryo fibroblasts (MEF) revealed that many epithelial and pro-apoptotic genes are de-regulated, suggesting an important role of CtBP proteins during epithelial to mesenchymal transitions, potentially contributing to tumor malignancy.[12]

Although the impact on the CtBPs on hematopoiesis has not been examined in detail, the microarray study on MEF showed evidence that several hematopoietic genes are up-regulated in the absence of CtBPs (Table 1). Several of the genes that were found to be dys-regulated are erythroid genes that are normally up-regulated by GATA-1 during erythroid differentiation.[13] The GATA cofactors, FOG and FOG-2 are known to bind CtBP (see below). Although GATA-1 and FOG are erythroid proteins and are not likely to be present in MEF, it is likely that other GATA and FOG family proteins, such as the more broadly expressed GATA-2 and FOG-2 are present. Thus the apparent up-regulation of the erythroid genes raises the possibility that GATA and FOG proteins are involved in the repression of ectopic expression of erythroid genes in nonerythroid tissues. It also suggests that GATA-1 and FOG may be involved in repressing some of these genes in early stages of erythroid development. Indeed strong roles for GATA-1 in gene repression have recently been detected in microarray experiments.[13] Further experiments, such as the generation of mice harboring blood lineage

selective mutations in both CtBP1 and CtBP2, will be required to decipher the full role of these corepressors in hematopoiesis.

CtBP Partnership with Hematopoietic Factors

CtBP proteins are known to interact with a short sequence motif (PXDLS) present in a variety of transcription factors and cofactors.[11,14] Among them, FOG, BKLF and Ikaros are known to play key role during hematopoiesis. The functional interactions with CtBP proteins are reviewed in this section.

GATA and Friend of GATA (FOG) Proteins

FOG (Friend of GATA-1) was identified in a yeast two-hybrid screen as a GATA-1 cofactor.[15] FOG contains nine zinc fingers of two different types, C_2H_2 and C_2HC, distributed throughout the protein (Fig. 1). When hematopoietic cell lines were examined, it was found that FOG displayed an expression pattern strikingly similar to that of GATA-1. That is, FOG is found in erythrocytes, megakaryocytes and multipotential progenitors. Moreover, FOG-1-/- mice die between E10.5 and E12.5 from severe anemia and exhibit a block in erythroid maturation at a stage similar to that observed in GATA-1-/- mice and also a complete failure of megakaryopoiesis.[16] These results provide strong genetic evidence that FOG and GATA-1 function in a coordinate manner in erythroid development (Fig. 2).[2]

Searches of the murine expressed sequence tag (EST) databases revealed the presence of a second FOG gene named FOG-2.[17,18] FOG-2 is expressed highly in heart, brain and liver and mirrors the expression pattern of GATA-4/5/6, suggesting that FOG-2 may serve as a cofactor for these nonhematopoietic GATA factors. The phenotypes of mouse embryos deficient in FOG-2 support this view. They die between E12.5 and E15.5 due to a complex congenital cardiac defect.[19] A structurally FOG-related protein, U-shaped (Ush) is present in *Drosophila*, whereas a single FOG gene has been identified in *Xenopus* and three in *Zebrafish*.[2]

FOG can function as either a transcriptional coactivator or repressor depending on the cell and promoter context.[20,21] Although the mechanism of repression by FOG proteins remains elusive, numerous studies suggest that the corepressors CtBP may contribute to repression. Indeed, a common feature among all identified FOG proteins is the presence of a PXDLS motif (Fig. 1) and mutation of this motif consequently abolishes FOG/CtBP interaction.[6,21,22]

Experimental evidence supporting a role for CtBP proteins in contributing to FOG activity has come from experiments with mFOG and mFOG-2 in *Xenopus*. Ectopic expression of mFOG and mFOG-2 in *Xenopus* blocks erythropoiesis and reduces *xGata-1* and *xSCL* levels, suggesting that FOG proteins limit red blood cell formation to prevent depletion of pluripotent cells.[23] Conversely, expression of FOGΔCtBP (a FOG mutant unable to interact with CtBP) augments red cell production in whole embryos, arguing that FOG proteins require the CtBP corepressors to regulate lineage commitment in this system.[23] In agreement with this, rescue of FOG-1-/- mouse cell line with a FOGΔCtBP mutant resulted in a marked enhancement of erythropoiesis, compared to that achieved by wild-type FOG.[22] This result suggests that CtBP proteins play a role in tempering the ability of FOG proteins to drive erythropoiesis. In stark contrast to these results, however, erythropoiesis appears normal in FOGΔCtBP knock-in mice.[22] The discrepancy between *Xenopus* and cell line experiments and the mouse knock-in experiment is puzzling but may be due to compensatory mechanisms in developing mice that are not available in the other systems, or it is also possible that the knock-in mice have subtle defects not yet detected.

A recent study suggests that FOG may impair the proliferation of hematopoietic cells in a CtBP-dependent and -independent manner, according to the differentiation stages.[24] In other words, CtBP proteins appear to repress erythropoiesis only in early stages but are not required in the late stages of cellular maturation. The fact that CtBP is expressed in G1E cells (an immortalized GATA-1 null line derived from gene-targeted embryonic stem cells) and down-regulated during GATA-1 reactivation supports this view and raises the possibility that

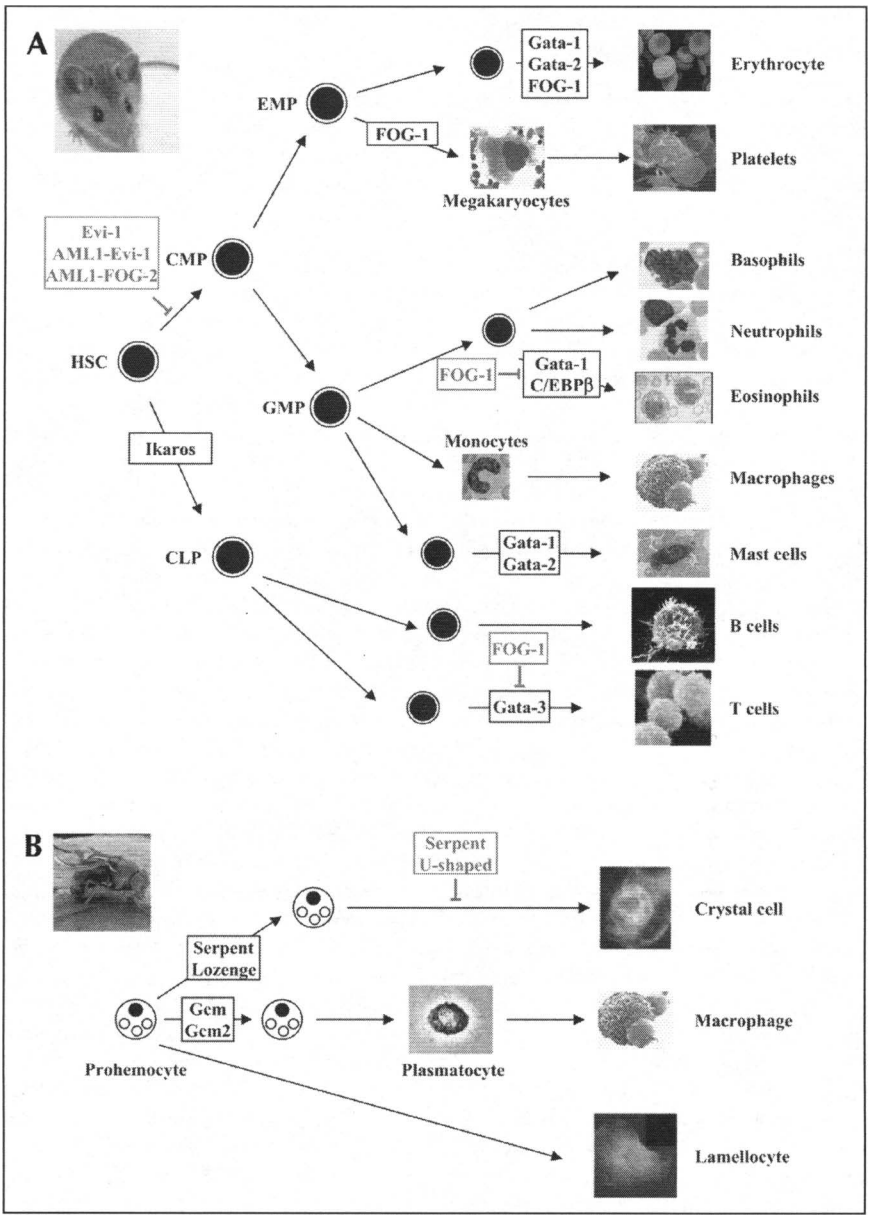

Figure 2. The hematopoietic tree. A) Schematic representation of the main lineage commitment steps in mouse hematopoiesis. The hematopoietic stem cell (HSC) gives rise to the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). CLPs give rise exclusively to B and T cells, while CMPs give rise to erythrocyte-megakaryocyte progenitors (EMP) and granulocyte-monocyte progenitors (GMP). Hematopoietic factors relevant for this chapter are indicated. Pictures are extracted from different web sites and available upon request. B) Summary of events during hematopoiesis in *Drosophila*. Hemocytes are derived from Glial-cells-missing (Gcm)-expressing prohemocytes. The GATA factor Serpent and the Runx factor Lozenge cooperate to give rise to crystal cell whereas Serpent and the FOG factor U-Shaped repress this pathway.

the regulation of CtBP level is important in the processes leading to erythroid maturation.[13] Recent work in *Drosophila* suggests that the requirement for CtBP reflects the need for overall higher levels of repression, rather than a requirement for an activity unique to CtBP,[25] strongly arguing for a quantitative rather than a qualitative CtBP repression function. According to this model, high level of CtBP in multipotent progenitors would limit the activity of GATA-1 but as differentiation and maturation proceeded, increases in GATA-1 levels combined with down regulation of CtBP would allow the activation of erythroid specific genes (Table 1).

As in vertebrates, hematopoiesis in *Drosophila* can be described as a biphasic developmental process that serves to populate the embryo, larva and adult with mature blood cells.[26] There are at least three terminally differentiated hemocyte types (Fig. 2): plasmatocytes that function primarily as phagocytes, crystal cells that function in the process of melanization and facilitate innate immune and wound-healing responses, and lamellocytes that appear to neutralize objects too large to be engulfed by plasmatocytes.[26] Interestingly, the *Drosophila* FOG homolog U-shaped (ush) is down-regulated during crystal cell lineage commitment, which is consistent with a role for the protein as a negative regulator of crystal cell production.[27,28] Furthermore ectopically expressed FOG suppresses crystal cell production in a CtBP-dependent manner.[29,30] It is noteworthy that CtBP is likely to be expressed during *Drosophila* hematopoiesis since it was found that a *lacZ* reporter gene inserted in the enhancer region of the CtBP gene is expressed in the larval plasmatocyte lineage.[31] Furthermore, a P-element based genetic screen designed to identify genes that control *Drosophila* hematopoiesis has also led to the isolation of CtBP.[32] Final evidence comes from the fact that mutations in CtBP alter the number of crystal cells. Surprisingly, however, unlike mutations in U-shaped, the mutations in CtBP cause a reduction in the number of crystal cells.

Interestingly, the disruption of eye development and the repression of cardiac cell development by Ush can occur in the absence of CtBP.[30] Similarly, mFOG and mFOG-2 can repress GATA-4 activation of cardiac promoters in a CtBP-independent manner.[33] Moreover, FOG is also able to repress GATA-3 activity during Th2 cell development[34] and C/EBPβ in eosinophil lineage commitment.[35] Whereas the repression of GATA-3 appears to be CtBP-independent, the role of CtBP on C/EBPβ has not yet been evaluated. Thus there are now a number of examples suggesting that while FOG proteins can recruit CtBP and CtBP can contribute to their repressive activity, the presence of CtBP is not required in all cases. In short, it is highly likely that FOG proteins bind other corepressors that can subsume CtBP's functions.

Another link between CtBP and GATA factors comes from work on the Mosquito *Aedes aegypti*.[36] The ingestion of blood is required for egg development in mosquitoes. In anautogenous mosquitoes, vitellogenesis is initiated only after a female mosquito ingests vertebrate blood. The blood meal triggers a hormonal cascade which activates yolk protein precursor (YPP) genes. A mosquito GATA factor called AaGATAr has been identified. AaGATAr serves as a transcriptional repressor to prevent the activation of YPP genes in previtellogenic females prior to blood feeding.[36] Interestingly, AaGATAr contains a PXDLS motif and thus its transcriptional repression appears to involve the recruitment of CtBP.[36,37] Thus, CtBP proteins appear to play a critical role in the repression of GATA-mediated activation through binding to FOG or through direct interaction with GATA, according to the localization of the PXDLS motif.

Krüppel-Like Factors

BKLF/KLF3 (Basic Krüppel-like factor/Krüppel-like factor 3) belongs to the mammalian Sp/Krüppel-like factor family, of which there are currently 24 members (Sp1-8 and KLF1-16). KLF proteins are characterized by a distinctive DNA binding domain at the C-terminus of the protein that consists of three Krüppel-like C_2H_2 zinc fingers. Outside this domain there is little homology among the known KLF proteins.[38] BKLF is highly abundant in erythroid cells and is known to function as a strong transcriptional repressor on several target promoters.[6,39] The repression domain of BKLF has been mapped to the N-terminal region and was found to associate with the transcriptional corepressor CtBP2 through the short CtBP interaction motif

PVDLT.[6] Disruption of the BKLF-CtBP interaction leads to a significant reduction of the repression potential of BKLF in cellular assays. In particular, BKLF represses GATA-1 activation of the erythroid EpoR and γ-globin promoters in a CtBP-dependent manner in gene reporter assays.[6,40,41] However, the functional significance of these interactions during development remains to be assessed in vivo.

Although the various KLF proteins share little overall homology outside their zinc finger regions, sequence alignments have shown that short stretches of homology do exist and the different KLF proteins can be grouped into subfamilies. One subfamily consists of BKLF/KLF3, AP2rep/KLF12 and KLF8. Although the homology between the repression domains is limited, it is significant that one prominent region of conservation encompasses the PXDLS motif used to contact CtBP corepressors. It has been shown that all three proteins can physically interact with CtBP through this motif and that the interaction is critical for gene repression.[6,42,43]

Ikaros

Ikaros is the founding member of a 'Greek' family of zinc finger DNA binding proteins that includes Aiolos, Helios, Eos and Pegasus.[44] Several of these transcription factors are thought to work in concert to promote the proper specification, differentiation and function of lymphocytes. The *Ikaros* gene encodes a protein with 6 zinc fingers that comply with the Krüppel C_2H_2 consensus arranged in two domains, the N-terminal domain involved in DNA-binding and the C-terminal domain involved in self-association (Fig. 1). Mice homozygous for an Ikaros mutation had no detectable lymphocytes or lymphocyte precursors (Fig. 2),[45] indicating that Ikaros is a critical player during lymphocyte development. Initial studies indicated that Ikaros was a weak activator of transcription but further reports clearly indicate that it is also a strong repressor.[46-48] In particular, Ikaros accumulates around clusters of centromeric heterochromatin.[49,50]

Ikaros interacts with a plethora of chromatin modifying enzymes including the Mi-2 histone de-acetylase complex.[51] HDAC association provided a likely mechanism for repression mediated by Ikaros[52] but HDAC-independent effects were also observed.[46] Importantly, a CtBP/Ikaros interaction is required for this HDAC-independent Ikaros-mediated repression.[46] Moreover, two other members of the family, namely Eos and the Ikaros related GATA protein TRPS1 (tricho-rhino-phalangeal type I) are can also recruit the corepressor CtBP to achieve strong transcriptional repression.[53] Taken together, these findings suggest that CtBP association is likely to be one of the mechanisms by which members of the Ikaros family of transcription factors mediate gene repression in vivo. However, establishing formally which genes are targets of either repression or activation by Ikaros family proteins and more precisely which genes are CtBP-dependent, will be important in defining the role of CtBP in lymphocyte commitment.

CtBPs in Leukemogenesis

Early studies indicate that deletions within the C-terminal region of the Adenovirus E1A protein, the region that encompass the conserved CtBP binding motif, confer a hyper-transforming phenotype to E1A. This enhanced activity is seen in assays when E1A is used in cooperation with the activated *Ras* oncogene to drive transformation.[4,54-56] Tumors expressing the E1A mutants are also highly metastatic, further suggesting that CtBP may attenuate the oncogenic potential of E1A. Recent results, however, also suggest that CtBP may contribute to oncogenesis, especially in blood cells.

Evi-1 (Ecotropic viral integration site 1) was initially identified as a common locus of retrovirus integration in myeloid tumors in AKXD mice.[57] Evi-1 is a transcriptional regulator that possesses two clusters of C_2H_2 zinc fingers (Fig. 1) and is implicated in myeloid leukemogenesis. In addition, a t(3;21)(q26;q22) translocation found in chronic myeloid leukemia cells generates a fusion transcript that contains AML1 linked to Evi-1.[58] Interestingly, Evi-1 contains a

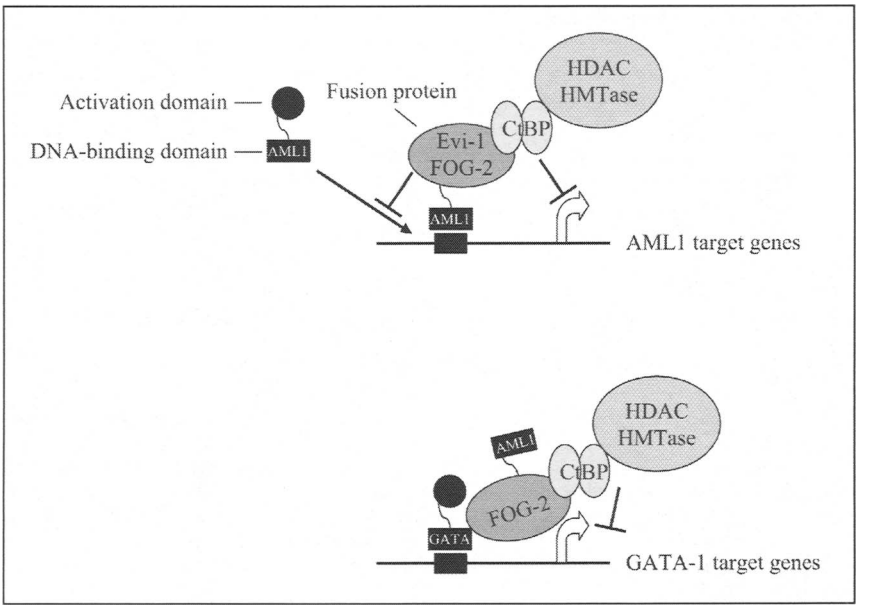

Figure 3. CtBP in leukemogenesis. Two models for transcriptional repression of AML1 or GATA-1 targets genes are shown in which AML1/Evi-1 or AML1/FOG-2 repress transcription by aberrantly recruiting the CtBP corepressor complex containing Histone deacetylase (HDAC) and Histone methyltransferase (HMTase) activity, respectively. Adapted from references 58 and 66.

repression domain with two CtBP binding motifs and mutations in these motifs impair transcriptional repression.[59-61] Moreover, these mutations also impair transformation of rat fibroblasts in vitro,[60] suggesting that CtBP plays a role in Evi-1 mediated leukemogenesis.

Several mechanisms have been proposed to explain how AML1/Evi-1 fusion proteins bring about the malignant transformation of hematopoietic stem cells.[58] For example, the AML1/Evi-1 fusion proteins are thought to exert a dominant negative effect and repress AML1 target genes by recruiting a CtBP corepressor complex through the Evi-1 part of the chimera[62,63] (Fig. 3). Remarkably, studies carried out with other fusion protein such as PML/RARα support the hypothesis that the inappropriate recruitment of corepressors has a causative role in the promotion of leukemia.[64,65]

Furthermore, CtBP has been recently been implicated in a newly characterized t(X;21)(p22.3;q22.1) translocation found in a patient with myelodysplasia that fuses AML1 with FOG-2.[66] Preliminary results suggest that as seen with AML/Evi-1, the AML1/FOG-2 fusion is able to recruit CtBP and thus may repress AML1 and GATA target genes (Fig. 3).[66]

CtBP proteins also bind the repression domain of MLL (mixed-lineage leukemia), a well known factor involved in more than 30 different fusions in leukemogenesis.[67,68] Finally, abnormal expression of CtBP has been proposed to contribute to development of Hodgkin's lymphoma.[69] In summary, these studies clearly indicate that CtBPs proteins are implicated in pathways leading to abnormal hematopoeitic growth and differentiation.

Conclusion

CtBP proteins interact with a myriad of transcription factors involved in many key developmental processes. There is now considerable evidence that CtBP physically and functionally interacts with several proteins that play key roles in hematopoietic development. Thus it seems likely that CtBP will play important roles in the control of hematopoietic development.

Nevertheless there is also clear evidence that CtBP is not always an obligate partner of hematopoietic regulators. It is possible that it is required to repress certain target genes during specific stages of development but that it is dispensable on other target genes or in other cellular contexts. The CtBP-independence observed may in some cases indicate that it is simply not required and in other cases it may be that another corepressor subsumes the role of CtBP. Further experiments will be required to delineate the precise roles of CtBP during hematopoiesis and to identify the specific subset of genes that it regulates. This information may prove critical to the ability to artificially control hematopoiesis and to treat disorders such as leukemias that arise from inappropriate gene control during blood development.

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CHAPTER 4

CtBP:
A Link between Apoptosis and the Epithelial-Mesenchymal Transition

Steven M. Frisch*

Abstract

Adenovirus E1a proteins are potent and ubiquitously acting tumor suppressors in human tumor cells. Through interaction with CtBP (as well as other mechanisms), E1a protein sensitizes cells to several apoptotic responses including anoikis. This interaction also induces the expression of certain epithelial cell adhesion and cytoskeletal genes in various tumor cell lines. Functionally analogous results are observed in mouse embryo fibroblasts lacking CtBP1 and CtBP2 genes. These results implicate CtBP as a potential modulator of the epithelial-to-mesenchymal transition (EMT) as well as apoptosis.

Introduction

The epithelial-to-mesenchymal transition (EMT) is an important feature of embryonic development as well as the evolution of carcinoma cells, but the relationship between EMT and the latter has, until recently, been somewhat phenomenological: cadherin/catenin signaling and cell polarity are deregulated, which somehow promotes tumor progression.[1]

More recently it has become appreciated that carcinoma cells are generally deficient in multiple apoptotic signaling pathways. In particular, their sensitivity to *anoikis*—apoptosis triggered by detachment from matrix, or attachment to the wrong matrix—is compromised (reviewed in ref. 2). The oncogenicity of EMT can now be explained because anoikis is a general feature of epithelial but usually not mesenchymal cells, so EMT programs anoikis-resistance, thus promoting tumor progression.

These combined observations frame an important question: is there a mechanistic link between the EMT and the acquisition of apoptosis-resistance? Namely, is there a specific factor or family of factors that regulates these two gene expression programs coordinately? The protein that is the subject of this book, C-terminal Binding Protein (CtBP) appears to have the properties of this factor. These properties and the indications that CtBP might represent a novel cancer drug target are summarized in this chapter.

Discovery of CtBP's Phenotypic Properties in Human Tumor Cells Using E1a as a Probe

Even though the adenovirus E1a 243 amino acid protein is oncogenic in rodent cells—primarily due to its inactivation of the retinoblastoma protein—E1a is decidedly

*Corresponding Author: Steven M. Frisch—Mary Babb Randolph Cancer Center and Department of Biochemistry, West Virginia University, 1 Medical Center Drive POB 9300, Morgantown, West Virginia 26501, U.S.A. Email: sfrisch@hsc.wvu.edu

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tumor-suppressive in human tumor cells due to multiple effects mediated by several protein interactions (reviewed in ref. 3).

Interestingly, E1a expression induces the expression of epithelial-specific cell adhesion and cytoskeletal genes in tumor cell lines of diverse origin, including melanoma, fibrosarcoma, rhabdomyosarcoma and others.[4] This is accompanied by the sensitization of certain cell lines to anoikis.[5,6] Mutant E1a proteins that fail to bind CtBP are partially defective in both of these effects.[5]

These results suggested that the corepressor CtBP tends to target certain epithelial-specific and apoptosis-promoting genes for repression. Indeed, our microarray analysis[7] of cells from the CtBP1,2-double-knockout mice indicated that there was such a tendency. Many other genes were regulated as well, but the only recognizable *programs* of genes that were *coordinately* induced by CtBP1,2 double knockout were epithelial-specific genes (e.g., cytokeratins and cell junction proteins), as well as pro-apoptotic genes such as PERP—which is pro-apoptotic, p53-inducible gene encoding a desmosomal protein[8,9]—and the BH3 domain protein, Noxa. CtBP is thus a corepressor for epithelial as well as pro-apoptotic gene expression, with properties of a master regulator that links these programs.

One caveat is that CtBP is known also to function as a corepressor for a very wide variety of repressor proteins, targeting lymphoid, muscle or neuronal-specific genes. This raises the question of why these genes usually are not also induced by the genetic or E1a-mediated inactivation of CtBP. A partially speculative answer is that epithelial gene promoters such as the E-cadherin promoter (which is a known target for repression by CtBP-ZEB1 and CtBP-SIP1 complexes) appear not to require tissue-specific transactivator proteins for expression, needing only ubiquitous factors such as Sp1, NF1/CTF, and others, for expression (although a novel intron 2 enhancer has recently been identified, which may potentially interact with additional activators or repressors, as yet unidentified[10]). We hypothesize that because the transactivators needed to drive their expression are ubiquitous, these promoters (in contrast with other tissue-specific promoters, e.g., muscle) are induced by the simple removal of CtBP-repressor complexes. Whether an analogous phenomenon can be generalized across the many epithelial and pro-apoptotic gene promoters that are induced when CtBP is lost remains to be seen. In the most stringent application of this model, though, "induction by loss of CtBP" may define those genes that share the simplicity of transcription factor requirements with E-cadherin.[11] Repressors for the E-cadherin promoter include Snail[12,13]and Slug[14]—which are not known to interact with CtBP (in mammalian cells)—as well as ZEB1/deltaEF1[5] and ZEB2[15] which repress transcription partly by recruiting CtBP; in some cell systems Snail induces ZEB1 expression in addition.[16] E1a is thought to de-repress the promoter by interfering with ZEB-CtBP interaction, although additional mechanisms may play a role.

Regulation of CtBP Function by Kinases and Sumoylation: Implications For Gene Expression and Apoptosis

Analogously with the control of coactivator function by kinases, the corepressor function of CtBP protein is regulated by several modifications affecting its localization, repressor interaction and degradation.

With regard to localization, CtBP has been variably reported as entirely nuclear or as partly cytoplasmic; indeed a cytoplasmic function in Golgi tubule dynamics has been proposed.[17] Thus, the localization of CtBP may prove to be somewhat cell-type-, treatment-, antibody- or detection method-dependent. Nevertheless, a recent report demonstrates that a fraction of CtBP protein is sumoylated by PIAS proteins in vitro and in vivo, and that mutation of the Sumo acceptor site (K428) virtually abolished its nuclear localization.[18] Conversely, binding of cytoplasmic PDZ-containing proteins such as nNOS to CtBP's PDZ binding domain adjacent to the Sumo acceptor site (DQL438-440) blocked Sumoylation and resulted in the cytoplasmic accumulation of CtBP. It is difficult to reconcile that only a small fraction of CtBP protein is sumoylated, while most of the CtBP is nuclear, with the simple model proposed. However, it

is conceivable that CtBP protein requires Sumoylation only for nuclear import but not for stable retention in the nucleus. Alternatively, a second report demonstrates an important role for the polycomb repression complex protein Pc2 as an E3 ligase protein that Sumoylates CtBP and recruits it to polycomb foci.[19] This represents an intranuclear rather than a nuclear vs. cytoplasmic redistribution of CtBP. These important details need to be addressed and reconciled. Nevertheless, these observations imply that Sumoylation of CtBP may be an important regulatory step, analogous to that in other nuclear apoptosis regulators such as p53, PML and DAXX. Conceivably, the control of CtBP's sumoylation may play an important role in EMT and apoptosis regulation.

Interestingly, CtBP interacts with and is phosphorylated by p21-activated kinase (PAK), an integrin- and growth factor receptor-activated kinase that translocates to the nucleus.[20] This phosphorylation (S158) is reported to cause CtBP to accumulate in the cytoplasm, thus preventing corepression; siRNA-mediated PAK1 depletion resulted in almost exclusively nuclear localization. The phosphorylation occurred preferentially on the NADH-bound form of CtBP (see below). This mechanism predicts that the activation of PAK1 by cell adhesion signaling impedes the CtBP's gene repression activities, causing a pre-apoptotic effect. This is paradoxical because cell adhesion signaling, PAK1[21] and CtBP[5,7,22] are usually considered to be "pro-survival". These results can however, be reconciled if: (i) it is primarily a pro-apoptotic fragment of PAK (e.g., the caspase-activated form of PAK2[23] that is responsible for CtBP phosphorylation, or (ii) CtBP regulates apoptosis vs. gene expression independently—a possibility that will be addressed below.

Another interesting connection between integrin/cytoskeletal signaling and CtBP is that E1a induces the expression of Tiam-1, a Rac activator.[24] Intriguingly, either Tiam-1 over-expression or E1a expression caused reversal of EMT in ras-transformed MDCK cells, accompanied by restoration of epithelial morphology and junctional complex assembly. The effect of E1a was abrogated in cells where Tiam-1 was depleted. These results suggest that E1a induces Tiam-1 expression (possibly through interaction with CtBP). According to the model proposed above, this would activate PAK activity through Rac, providing a second mechanism of inactivating CtBP—possibly by relocalization—causing reversal of EMT. It will be relevant to determine whether Tiam-1 acts similarly to PAK1 over-expression in its effects upon CtBP.

In connection with aforementioned idea that CtBP could regulate transcription and apoptosis independently, certain apoptotic stimuli such as ultraviolet light have been found to cause the rapid degradation of CtBP, due to the phosphorylation of CtBP1 on S422 by homeodomain-interacting protein kinase-2 (HIPK2), a highly pro-apoptotic kinase that also activates p53.[22] This phosphorylation triggers poly-ubiquitination and proteasome-mediated CtBP degradation. In certain cell lines (HeLa, H1299), siRNA-mediated partial depletion of both CtBP1 and -2 induced a detectable degree of caspase-3 induction and apoptosis.[22] These results can be interpreted in terms of de-repression of "toxic genes" such as Noxa, PERP and Bax, resulting in cell death. Alternatively, there could be a direct interaction between CtBP and some apoptosis-regulatory factor, or the absence of CtBP could create an aberrant chromatin structure that the cell interprets as DNA damage, engaging a DNA damage checkpoint response (although this does not occur in T-antigen immortalized mouse embryo fibroblasts lacking CtBP). It remains to be determined whether the degradation of CtBP triggers apoptosis mainly through transcriptional vs. direct apoptotic signaling mechanisms.

In either event, the inactivation of CtBP by degradation in response to HIPK2 activators such as UV light or by the lack of the required cofactor NADH (see below) would be predicted also to cause the upregulation of epithelial-specific genes such as E-cadherin—as well as a reversal of EMT—an intriguing possibility that remains to be addressed.

Regulation of CtBP Function by NADH

A series of elegant biochemical, biophysical and in vivo studies from the Goodman laboratory has revealed that in order to bind repressors and repress transcription efficiently, CtBP

must interact with NADH;[25,26] in addition, it has been reported that NADH or NAD+ stimulate CtBP oligomerization,[27] raising some degree of controversy as to whether the effect of NAD is specific to the reduced form (although only one concentration of NAD+ and NADH was used in this latter study, in contrast with the previous studies which compared concentration-dependence curves). These results potentially reveal a new insight into how the redox state of the cell influences apoptosis and gene expression. For example, the NADH/NAD ratio of tumor cells is frequently abnormally high[28-30] due to hypoxia,[31] p53 deletion[32] or bcl-2 over-expression.[33,34] At these higher ratios, CtBP is predicted to be maximally functional, thus conferring apoptosis-resistance and promoting EMT—common signatures of tumor cells. Although this hypothesis remains to be addressed, there is already evidence that the E-cadherin promoter is repressed more efficiently by NADH-CtBP complexes than by CtBP alone,[26] and, independently, there are reports that hypoxia can repress endogenous E-cadherin expression.[35]

Conclusions and Perspectives

By binding CtBP in tumor cells, E1a promotes epithelial-specific gene expression and apoptosis-sensitivity, contributing to E1a's tumor suppression effect. However, this does not necessarily imply that CtBP acts oncogenically in all of its diverse activities, and, in fact, CtBP was recently shown to antagonize the Wnt pathway by binding to APC protein and sequestering beta-catenin away from TCF factors [36] (implying a tumor suppressive activity for CtBP in this context). Nevertheless, it is likely—based on the effects of E1a and CtBP knockout—that the net consequence of CtBP inactivation in tumor cells will usually be tumor suppression. Is there a cellular protein that inactivates CtBP analogous to E1a? A recent report demonstrates that the RNA splicing factor/desmosomal protein Pinin (Pnn) interacts with CtBP, inhibiting the corepression function of the latter.[37] Accordingly, Pnn over-expression induced the E-cadherin gene. Pinin was found to induce epithelial cell adhesion molecule genes and to inhibit cell motility and anchorage-independent growth (perhaps through induction of anoikis-sensitivity);[38] its expression is decreased in various human tumors, partly due to promoter methylation, suggesting that it may prove to be a tumor suppressor gene.[39] It will be interesting to analyze gene expression and apoptosis in Pnn-over-expressing cells to compare the effects of Pnn with E1a.

Acknowledgment

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CHAPTER 5

The Significance of the CtBP – AdE1A Interaction during Viral Infection and Transformation

Roger J.A. Grand,* Claire Baker, Paola M. Barral, Rachel K. Bruton, Julian Parkhill, Tadge Szestak and Philip H. Gallimore

Abstract

C-terminal binding protein (CtBP) associates with adenovirus early region 1A (AdE1A) proteins through a highly conserved PXDLS motif located very close to its C-terminus in conserved region 4. To try to understand the importance of this interaction for the virus a point mutation in the CtBP binding site of Ad12E1A (P→S at amino acid 255) was engineered. The mutant Ad12E1A DNA (Ad12E1A6f) encoded a protein temperature sensitive (ts) for transformation of baby rat kidney cells when in combination with Ad12E1B. At 33°C transformation frequency was comparable to *wt*. At 37° and 38.5° transformants appeared as larger epithelioid cells and colonies senesced relatively rapidly. When the Ad12 6f AdE1A was incorporated into a mutant virus it caused a marked reduction in its ability to replicate with only Ad12E1A and Ad12E1B19K being expressed at early times. It was observed that 6fE1A bound to CtBP very inefficiently. Ad12E1 transformed rat cell lines, carrying the 6f mutation were established from the 33°C transformants but failed to express the Ad12E1B54K protein. After a number of weeks in culture the cells developed a mesenchymal character; expression of proteins such as E-cadherin, P-cadherin and γ catenin was much reduced and expression of fibronection increased. These observations are consistent with inhibition of CtBP activity in *wt* Ad12E1 transformants but not in the 6f transformed cells. In a complementary study the effect of down-regulation of CtBP expression (using siRNA protocols) was examined. Consistent with results obtained with the 6f virus it was observed that reduction in expression of CtBP1 and CtBP2 facilitated viral infection and this effect was enhanced when expression of C-terminal interacting protein (CTIP) was also reduced.

Introduction

Adenovirus early region 1A (AdE1A) is the first viral protein to be expressed following viral infection and is essential for Ad-mediated transformation of mammalian cells in culture.[1,2] AdE1A's primary, although not only, role is as a regulator of transcription and it is through this activity that it can drive expression of other viral early region genes and usurp the cellular mechanisms of growth control.[3,4]

*Corresponding Author: Roger J.A. Grand—Cancer Research U.K. Institute for Cancer Studies, University of Birmingham, Birmingham B15 2TT U.K. Email: R.J.A.Grand@bham.ac.uk.

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As AdE1A appears to possess no enzymic activity and is unable to bind to DNA it is likely that all its activities are dependent upon interaction with cellular proteins.[1,5,6] The binding sites for these are distributed throughout the E1A molecule although most are concentrated within the N-terminal region and those amino acid sequences which are most highly conserved between AdE1As from different virus serotypes.[7-9] There are considered to be four conserved regions (CRs), with, for example, CR1 and CR2 containing binding sites for the Rb family of proteins;[10,11] CR1, together with the N-terminal region, interacting with CBP/p300[12] and CR3 binding to a variety of proteins involved in transcriptional activation such as TBP, transcription factors and TAFs.[13] Conserved region 4 has, in the past, been most notable for interaction with C terminal binding protein (CtBP) but more recently it has been shown to contain binding sites for Dyrk and $p27^{kip1}$.[14,15] The N-terminal region is involved in the association of AdE1A with regulatory components of the proteasome,[16] with p400 and TRRAP-containing complexes[17,18] and with TBP.[19] Space precludes a detailed consideration of the multiple interactions of AdE1A[1,2] but it is notable that binding to certain partners can be associated with particular biological properties of E1A. For example, interaction with the Rb family and CBP/p300 is necessary for initiation of cell cycle progression during viral infection and for cellular transformation.[20-22] Similarly binding to transcription factors and the basic transcriptional machinery through CR3 is required for the expression of other viral early region genes (reviewed see ref. 13). A further host cell binding protein which appears to be of considerable significance in determining the activities of AdE1A is CtBP-a transcriptional corepressor which interacts with a highly conserved motif occurring in CR4 very close to the C-terminus of virtually all AdE1As.[8,9,14,23]

CtBP was first isolated on the basis of its ability to bind to exon 2 of AdE1A.[14] The essential site of interaction on AdE1A comprises a short amino acid sequence, PXDLS, now known to be widespread in CtBP binding proteins.[23-26] However, considerable effort has been devoted to understanding whether amino acids outside the PXDLS sequence can contribute to the binding motif. Data derived from a study of synthetic peptides and AdE1A protein domains certainly suggest that this might be the case. For example it has been shown that substitution of amino acids outside the PXDLS site causes changes in Kd of peptides for CtBP.[27] Similarly, synthetic peptides with identical PXDLS motifs but with different surrounding sequences can have different K_i for the inhibition of Ad12E1A binding to CtBP1.[28] Furthermore it appears that full-length Ad12E1A will bind more strongly to CtBP than a polypeptide encompassing exon 2 (amino acids 190-266) and this, in turn, binds with higher affinity than a synthetic peptide comprising only 20 amino acids, but still containing PVDLS.[29] In addition it has been reported that mutations in exon 2 of AdE1A, outside the PXDLS motif, produce biological effects generally attributed to inhibition of CtBP interaction (compare refs. 14,30,31) CtBP appears to function primarily as a transcriptional corepressor. This may be through interaction with other coregulating proteins such as members of the human polycomb family,[32] *Drosophila* short range and long range repressors, such as Knirps, Snail and Hairy,[33,34] and/or through direct binding to histone deacetylases (discussed in more detail in refs. 24,26). It has been noted that HDAC-4 and HDAC-7 and perhaps HDAC-5 contain PXDLS motifs which are probably sites for CtBP binding but that HDAC-1, HDAC-2 and Sin3 interact in a PXDLS-independent manner.[15,35]

Two CtBP genes (1 and 2) have been mapped in mammals and these are approximately 80% homologous. Relatively little difference has been observed between the CtBP1 and CtBP2 proteins at the biochemical level but knock-out animals have appreciably different life expectancies.[36] CtBP1-/- mice are fertile but approximately 30% smaller than *wt* animals.[36] The CtBP2-/- mice die in utero at E10.5, possibly due to incorrect development of the placenta. It appears that these embryos also have defects in heart and neural development. A third CtBP protein (CtBP3) has been isolated from rat brain and been shown to possess acyl transferase activity.[37] This protein is, in fact, the product of alternate splicing of the CtBP1 gene, such that two proteins are coexpressed, differing only in the 12 N-terminal amino acids, in human,

mouse and rat (Barral et al, submitted for publication). Whether the mouse and human CtBP3 proteins have identical enzymic properties to rat CtBP3 is, at present, unknown.

Although the interaction of AdE1A with CtBP was the first to be described[14,23] the mechanism by which this advantages the virus during infection is still far from clear. AdE1A is able to induce expression of certain epithelial proteins such that cells expressing E1A adopt an epithelial phenotype.[38-40] It appears that the association of AdE1A with CtBP regulates this epithelial-mesenchymal transition (EMT). Thus a number of cellular genes are under negative regulation by CtBP and the introduction of AdE1A allows expression by de-repression. However, to what extent this occurs during viral infection is not clear. Evidence has also been presented to support the contention that CtBP influences AdE1A exon 1 activity.[41] When the N-terminal 90 amino acids of Ad5E1A were fused to a Gal4 DNA binding domain they were able to activate transcription through a Gal 4 binding site. The inclusion of the C-terminal domain of E1A repressed transactivation and this activity was attributable to the CtBP binding site.[41] It seems reasonable to suppose therefore that CtBP binding may affect the activity of proteins such as p300, CBP and P/CAF bound to the N-terminus, presumably through altering the secondary structure of AdE1A. Although the interaction of CtBP with AdE1A can modulate the activity of exon 1 it is also possible that proteins bound to the N-terminal region can regulate CtBP binding by acetylation of lysine 239 of Ad5E1A.[42] It appears that p300, CBP and P/CAF can all acetylate AdE1A, modulating its ability to bind CtBP and to de-repress gene transcription. Finally a direct association of CtBP with p300 has been described.[43]

The repression properties of the C-terminal region of AdE1A were reported some time before the isolation of CtBP.[30] It was shown that deletion of the C-terminal 67 residues of Ad5E1A increased the frequency of transformation of rat cells by AdE1A together with mutant *ras*.[14,30,44] Furthermore transformed cells, carrying E1A with a C-terminal deletion, were considerably more tumourigenic when injected into the syngeneic host or athymic nude mice.[14,30] It is clear therefore that binding of AdE1A by CtBP has a repressive effect such that AdE1A with a C-terminal deletion is considered to be "a supertransformer." In this case, co-transfection of Ad5E1A, with a C-terminal deletion, and E1B has an opposite effect, however, in that the inability to bind CtBP reduces the frequency of transformation.[45] Similarly, it appears that CtBP binding is required for induction of DNA synthesis and immortalisation of primary cells by Ad12SE1A.[46,47]

In the study presented here we have used an AdE1A mutant to assess the significance of the E1A/CtBP interaction during both viral infection and transformation, in the expectation that this may provide clues to the reasons why it is necessary for AdE1A to target this ubiquitous transcriptional corepressor.

The Phenotype of an Adenovirus 12 E1A CtBP Binding Site Mutant

Chemical mutagenesis of defined fragments of Ad12 E1A DNA has been used to screen for mutants with defects in virus transformation of primary baby rat kidney (BRK) cells. Mutagen-treated DNA was cloned to reconstitute E1A and E1B in *E. coli*. BRK transformation experiments were carried out at three temperatures, 33°, 37° and 38.5°C using unique plasmids. We screened 339 plasmids and of these 273 behaved as *wt* and 66 showed reduced or no transformation. 65 of these transformation - defective plasmids were either early termination mutants or multiple mutants (affecting 2 or more AdE1A amino acids). One plasmid, designated 6f, was found to have an unusual phenotype. At the two higher temperatures BRKs exposed to the 6f plasmid contained a significant proportion of viral transformants that were larger and flatter than those seen in cultures exposed to a plasmid containing wild type (*wt*) Ad12 E1 DNA (Fig. 1). With time, these abnormal 6f - induced foci stopped growing although the cells within the focus continued to increase in size and then took on a senescent phenotype. These senescent–like foci eventually (5-7 weeks post transfection) lost adherence and floated off into the tissue culture medium. On staining dishes at 7 weeks post-transfection there were significantly fewer transformed colonies on 6f dishes at the two higher temperatures

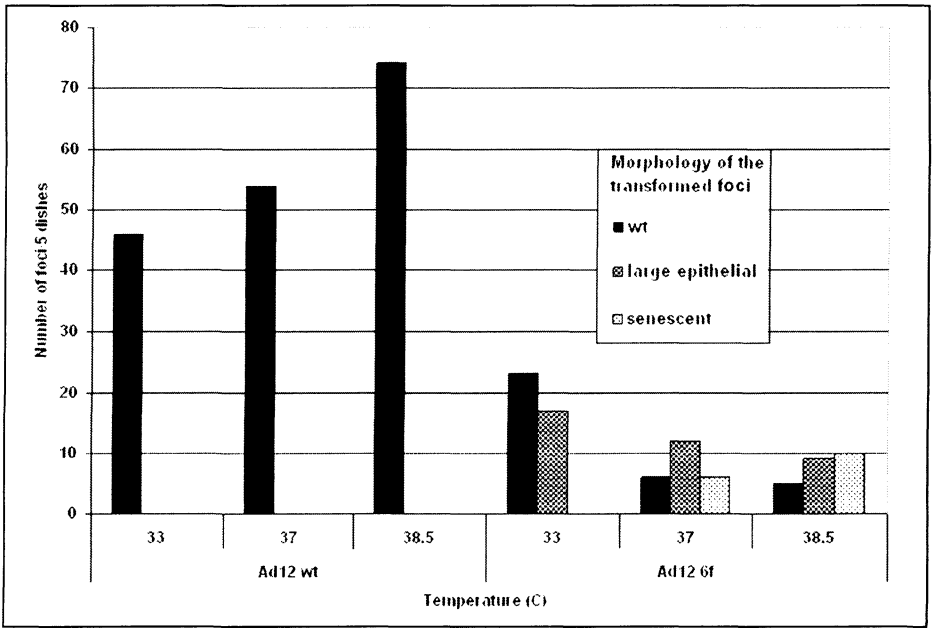


Figure 1. Frequency of transformation of BRKs by Ad12 *wt* and 6f E1A cDNA. HLBRKs were transfected with wild type and 6fAd12E1A together with Ad12E1B. Cells were incubated at 33° for 42 days and at 37°, and 38.5° for 21 days. Transformed foci were counted using low power microscopy. The data presented are representative of 6 independent experiments.

than in those cultures exposed to the *wt* Ad12 E1 plasmid. For BRKs maintained at 37° colony counts were 75% lower than *wt* but at 38.5°C they were down by an order of magnitude. Cell lines established from 6f viable colonies, grown up at 33°C and then switched to the highest nonpermissive temperature, revealed that these cells were partially temperature sensitive compared to their *wt* counterparts. Their growth rate was suppressed and plating efficiency reduced. However, once adapted to the higher temperature, the 6f cultures grew and plated as well as *wt* Ad12E1A - containing cells. Western blots shown in Figure 2, for two representative 6f cell lines and an Ad12E1 transformed baby rat kidney cell line confirm that the level of expression of Ad12E1A in all three is comparable. Similarly the three lines express very similar levels of Ad12E1B19K protein. However, expression of Ad12E1B54K is notably decreased in the 6f transformants; to such an extent that it is undetectable. These cells are, therefore, similar to Ad12 Hind III G transformants[48] and their morphology is consistent with this proposition. Western blotting for p53 indicates a relatively low level of expression in the 6f cells, compared to Ad12E1 transformants (Fig. 2) and thus provides further support for the suggestion that overexpression of p53 in Ad12E1 transformed cells is largely a function of Ad E1B 54K protein activity rather than AdE1A.[49]

Sequencing of 6f DNA revealed that a single base change had occurred at nucleotide 1338, (C→T) in Ad12 DNA incorporating serine at residues 255 (13S E1A mRNA) and 224 (12S E1A mRNA), a nonconservative change from the proline found in the Ad12 *wt* E1A polypeptides. Importantly, this mutation affects the first residue of the highly conserved PVDLS CtBP binding site. Previous studies of the effects of replacing each amino acid in the Ad12E1A CtBP binding motif (PVDLS) with alanine showed that the P→A substitution was the most detrimental to CtBP binding.[27] However when P was replaced with G the effect was not as marked.[27] It is not clear what quantitative effect the P→S mutation has on CtBP binding or on

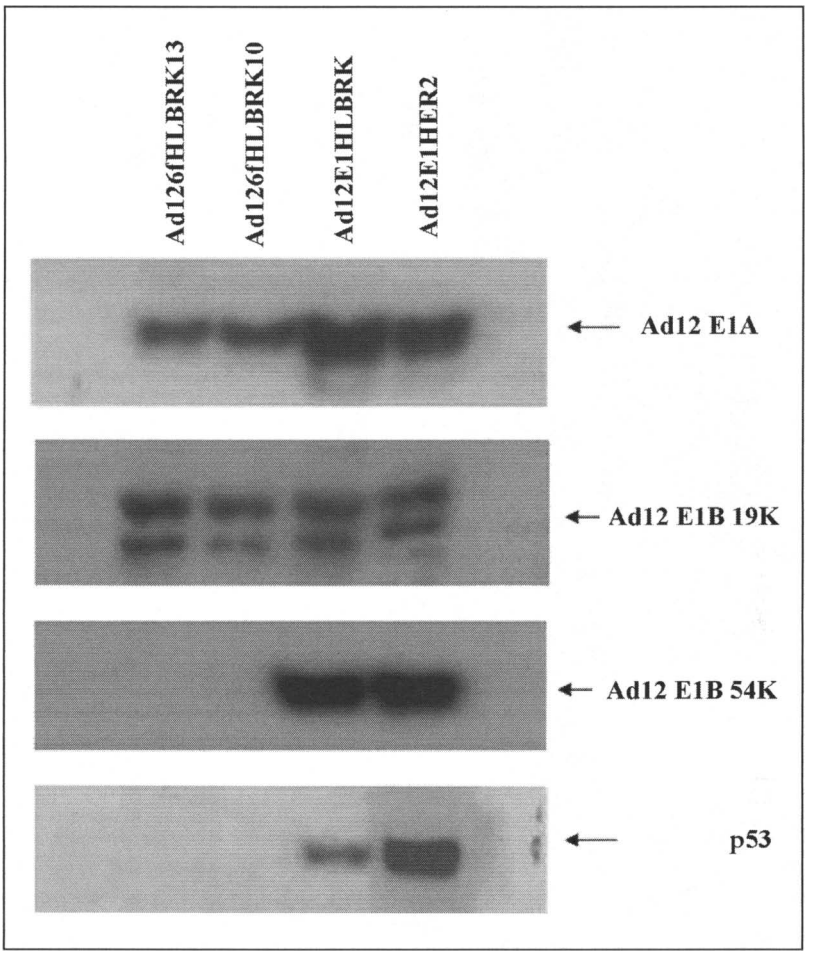


Figure 2. Expression of Ad12 early region 1 proteins in Ad126fE1 BRKs. Established AdE1HLBRK cell lines expressing Ad6fE1A were harvested, lysed and solubilized. After SDS.PAGE protein expression was monitored by western blotting. Aliquots (25 μg of total protein) of Ad12E1 HLBRKs, Ad126f HLBRKs (cell lines 10 and 13) and Ad12E1HERs were examined for Ad12E1A, Ad12E1B19K, Ad12E1B54K and p53 protein expression.

the structure of the C-terminal region. However there can be little doubt that the substitution appreciably reduces the ability of Ad12E1A to interact with CtBP (see below).

Using the same approach as Byrd et al,[50] the 6f plasmid was rescued into Ad12 virus and a number of plaque isolates sequenced across the entire E1 region. The majority of these contained the 6f mutation. One isolate was further plaque-purified and then grown up as a stock virus on Ad12E1 HER3 cells. This virus was designated as Ad12/6f and was examined for defects in virus infection and replication. Replication studies were carried out using two cell types, primary human embryo kidney cells (HEKs, the most permissive normal human cell host for *wt* Ad12 virus) and A549 cells (the most permissive human tumour cell line for Ad12 virus). At a multiplicity of infection of 50 plaque forming units per cell Ad12/6f was found to be disabled in both cell types when cells were incubated at 37° and 38.5°C; virus yields in HEKs were down by greater than three orders of magnitude and at least two orders of magnitude

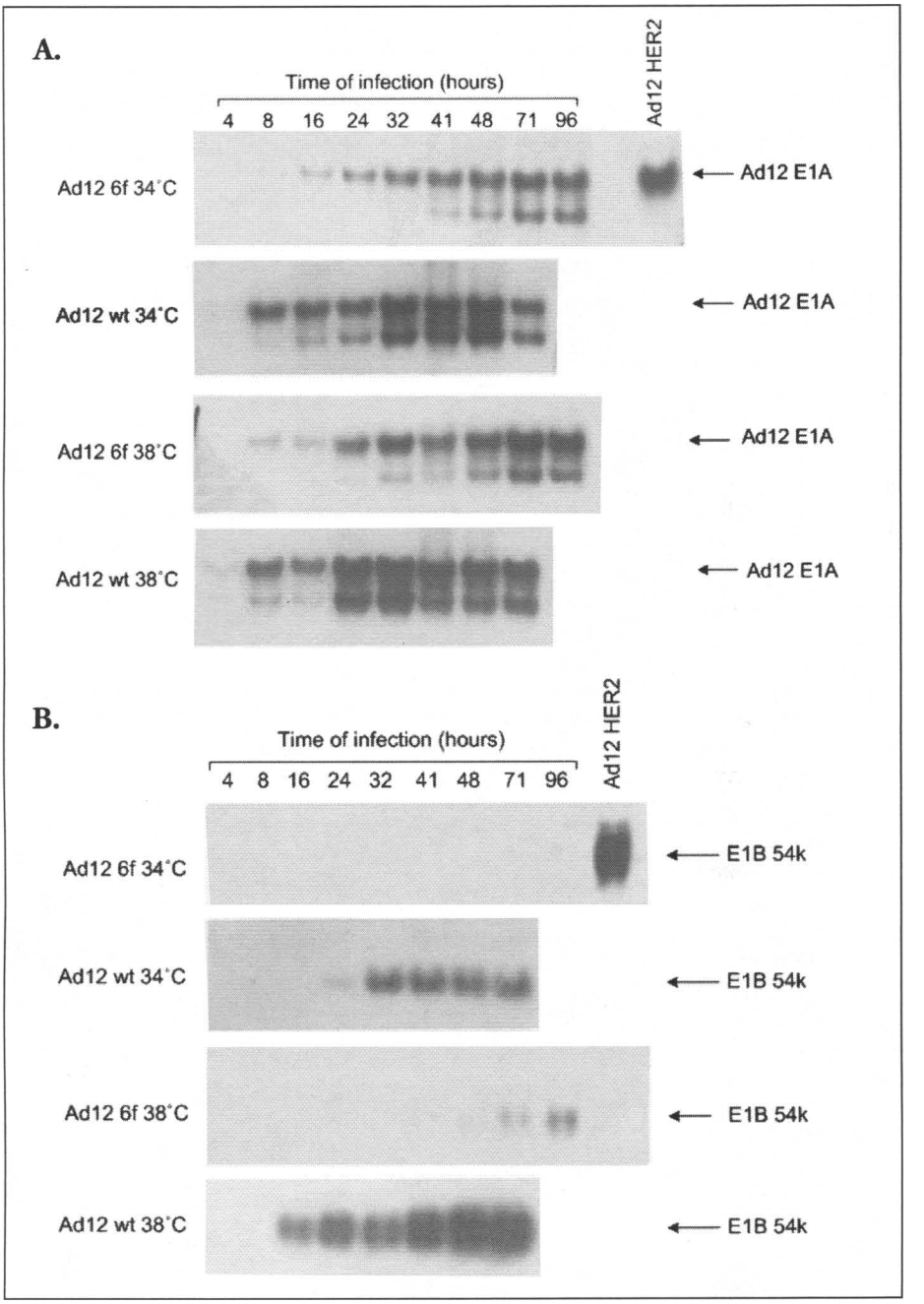
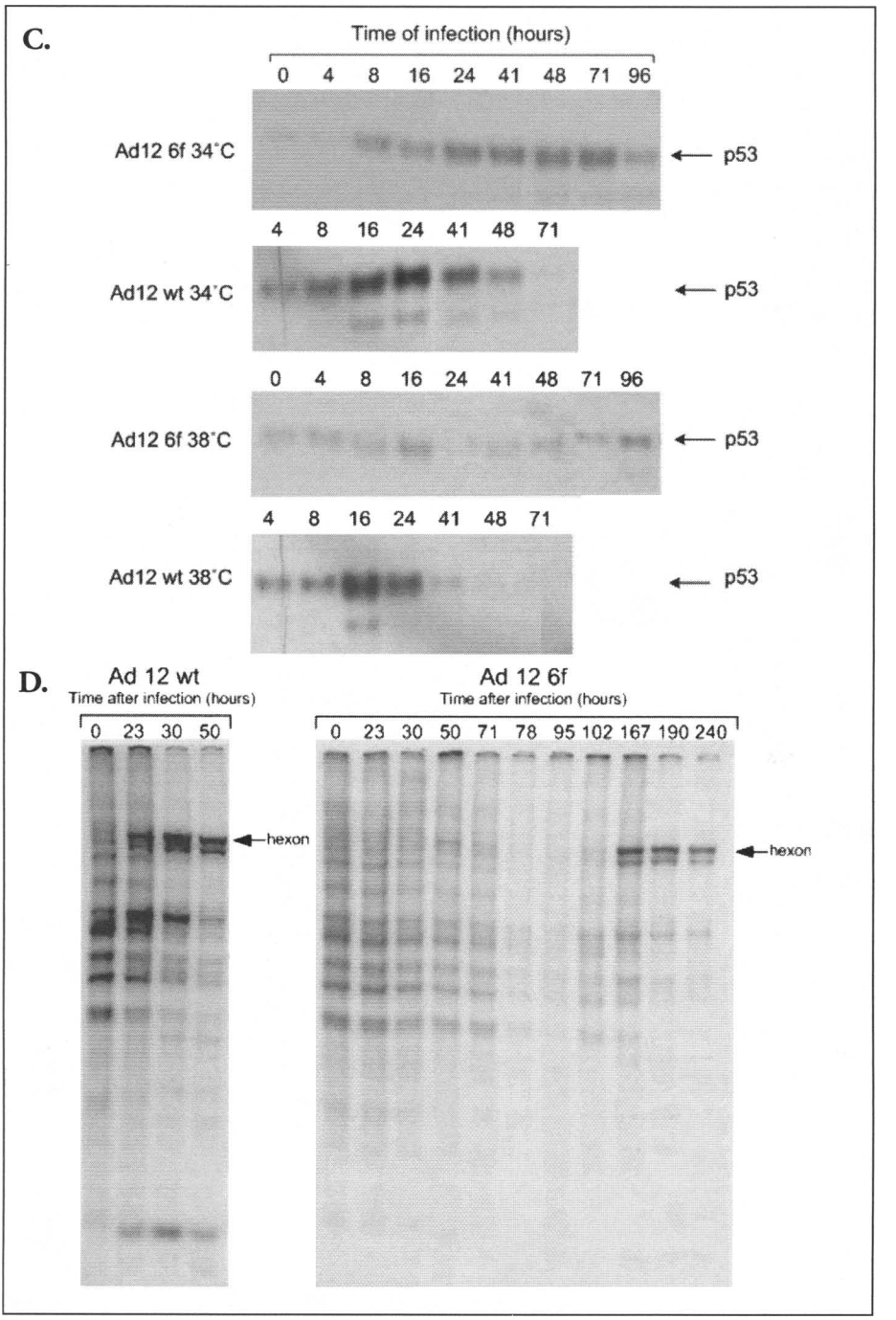


Figure 3. Infection of A549 cells with Ad12 *wt* and Ad12/6f viruses. A549 cells were infected with Ad12 *wt* and 6f virus at 34° and 38.5°C at an infectivity of 50 pfu/cell. After appropriate times cells were harvested, lysed and solubilized. Aliquots of proteins (25µg) were fractionated by SDS.PAGE. Ad proteins and p53 were detected by western blotting as shown; A) Ad12E1A; B) Ad12E1B54K; and C) p53. Panel D) expression of Ad12 structural proteins following infection with Ad12*wt* and Ad12/6f virus.



A549 cells were infected with virus (50pfu/cell) and then labelled with [^{35}S]-methionine (50 μCi/ml) after appropriate times for 1 hour in methionine-free medium. Cell lysates containing 10 μg of protein were fractionated by SDS.PAGE and radio-labelled proteins visualized by autoradiography.

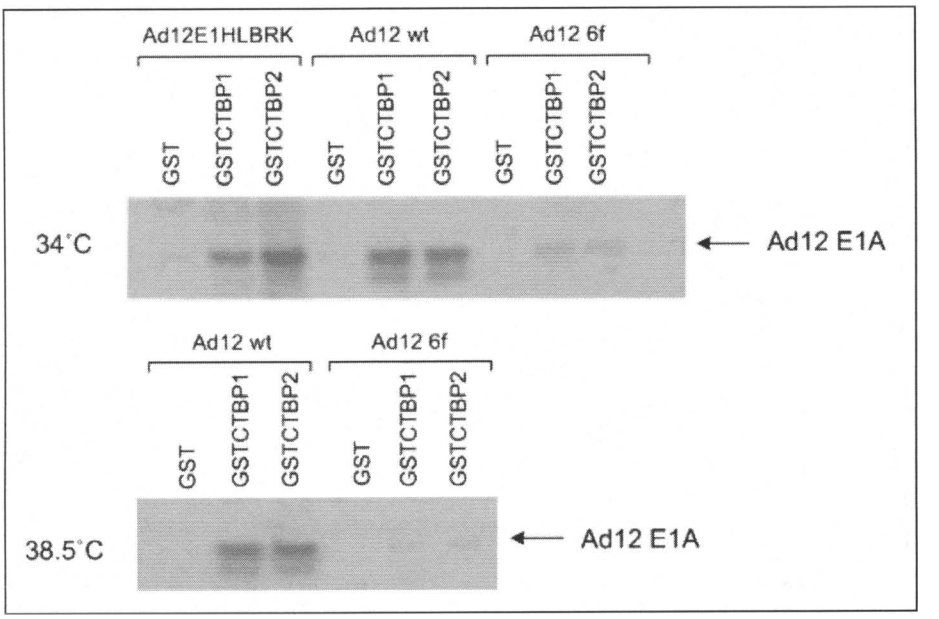


Figure 4. The interaction of CtBP with Ad12E1A and Ad126fE1A. A549 cells were infected with Ad12 *wt* and 6f viruses at 34°C and 38.5°C. After harvesting at 48 hours post-infection the level of expression of Ad12E1A was determined by densitometric scanning of western blots. Lysates containing equal amounts of Ad12E1A from *wt* and 6f infected cells at the two temperatures were incubated with GST, GST-CtBP1 or GST-CtBP2 (20 μg). Bound AdE1A was isolated using glutathione agarose beads and visualized by western blotting, using an antibody against Ad12E1A. Ad12E1 HLBRK cells are included as a positive control in the upper panel.

in A549 cells at the highest temperature. This is compelling evidence that CtBP binding plays a significant role in the outcome of an adenovirus infection. We then went on to examine viral protein expression following Ad12/6f infection of A549 cells and E1A/CtBP complex formation (Fig. 3).

Infection of Human Cells with Ad12/6f Virus

Comparison of the infectivity of human A549 cells with Ad12 *wt* and Ad12/6f viruses demonstrated clearly the marked effect of the mutation in the CtBP binding site. The western blots presented in Figure 3A, show that expression of Ad126fE1A is considerably reduced and can only be detected at later times. Furthermore appreciably less Ad12E1B54K protein could be seen following 6f infection (Fig. 3B). The results were comparable regardless of whether cells were infected at 34° or 38.5°C although viral infection proceeded more rapidly at the higher temperature. Despite the observation that the mutant AdE1A behaves differently, in a temperature dependent manner, in the transformation assays there is no evidence to support the contention that the protein adopts a wild type conformation at either temperature during infection. Western blotting for p53 showed reduced induction of expression by the 6f virus consistent with reduced AdE1A expression (Fig. 3C). In the case of both viruses however, the p53 was degraded at later times presumably through targeting to the proteasome by the E1B54K/E4orf6 complex. This seems to occur regardless of the reduced expression of E1B54K. To assess the expression of late viral structural proteins A549 cells were infected with Ad12*wt* or 6f and then proteins radiolabelled with [^{35}S]-methionine before harvesting at appropriate times. It can be seen that appreciable hexon expression occurred at 30 hours in the former case but only

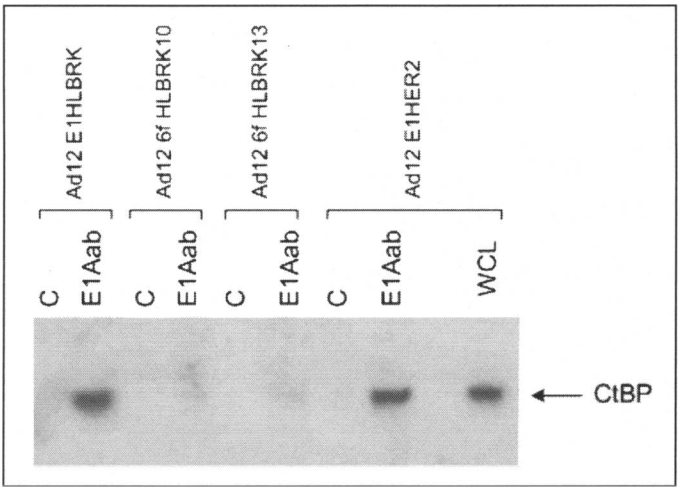


Figure 5. The interaction of Ad12E1A with CtBP in Ad12E16f transformed cells. Lysates from Ad12E1 HLBRK, Ad126f HLBRK 10, Ad126f HLBRK 13 and Ad12E1HER2 cells grown at 37° and containing equal amounts of protein were immunoprecipitated using a sheep antibody raised against Ad12E1A. Antigen-antibody complexes were isolated using protein G-agarose beads. Bound proteins were fractionated by SDS.PAGE and coimmunoprecipitated CtBP1 and CtBP2 identified by western blotting using a mouse monoclonal antibody. C: control irrelevant antibody; E1Aab: sheep polyclonal antibody against Ad12E1A; WCL: whole cell lysate.

after 167 hours in the latter, providing stark evidence of the detrimental effect of the AdE1A mutation on progeny virus production (Fig. 3D).

To determine the ability of Ad12 6fE1A to bind to CtBP during viral infection a series of pull-down experiments was carried out (Fig. 4). A549 cells were infected with Ad12*wt* and Ad12/6f at 34° and 38.5°C and harvested after 48 hours. Western blotting followed by densitometric scanning was used to determine the relative expression of mutant and *wt* AdE1A after infection at the two temperatures. Samples containing equal amounts of AdE1A were incubated with GST, GST-CtBP1 or GST-CtBP2 and then glutathione agarose. After extensive washing, bound AdE1A was detected by SDS.PAGE and western blotting (Fig. 4). It can be seen that appreciable *wt* Ad12E1A interacted with both CtBP1 and CtBP2. The mutant 6f protein, however, had a much lower affinity for both CtBP proteins. No real difference was observed between 6fE1A resulting from viral infections carried out at 34° and 38.5°C, although this is probably to be expected as, of course, the pull-down experiments were performed at 4°C.

Characterization of Rat Cells Transformed with Ad126fE1A

As mentioned previously it is possible to isolate transformed rat cell lines expressing Ad126fE1A and Ad12E1B19K protein (Fig. 2). Two of these lines (6f10 and 6f13) were studied in more detail. The level of expression of AdE1A was characteristically slightly reduced compared to an Ad12E1-transformed cell line although the level of the E1B19K protein was not (Fig. 2). No E1B54K could be observed. As has been noted previously[51] expression of p53 in adenovirus transformed rat cell lines, in the absence of the larger E1B protein, was very low. To confirm that mutation in the Ad12 6fE1A protein significantly impaired its ability to interact with CtBP in vivo coimmunoprecipitation experiments were carried out. Ad12E1A was immunoprecipitated using a sheep polyclonal antibody and bound CtBP detected by western blotting (Fig. 5). Very limited binding of the mutant E1A to CtBP was observed, consistent with the pull-down experiments presented in Figure 4. Further studies showed that when cell

lysates from Ad12E1A6f and Ad12E1 – transformed rat cells (grown at 34° and 38.5°C) were incubated with purified GST-CtBP1 and GST-CtBP2 little mutant E1A was bound compared to the *wt* protein (data not shown). However it should be noted that practical difficulties mean that it is not possible to regulate temperatures accurately during immunoprecipitation experiments, such that differences in binding at 34° or 38.5°C could not be detected.

It has previously been reported that CtBP1 plays a significant role in the regulation of expression of proteins that determine the epithelial cell phenotype and, indeed, may largely control epithelial-mesenchymal transition.[52] For example, CtBP represses expression of E-cadherin through interaction with the transcriptional repressor δEF1/ZEB.[53,54] Thus AdE1A induces human tumour cells to adopt epithelial characteristics through CtBP binding and inhibition of its activity.[38,52]

In view of these observations the expression of various cytoskeletal proteins, indicative of epithelial and mesenchymal phenotypes, was examined in two cell lines expressing the mutated (6f) AdE1A and this was compared with results obtained with *wt* Ad12E1A. Western blotting studies show a reduction in expression of E-cadherin, P-cadherin, N-cadherin and β-catenin in 6f transformants. This is accompanied by an increase in expression of β actin, paxillin and fibronectin (Fig. 6). These observations are consistent with the proposition that CtBP regulates the epithelial-mesenchymal transition (EMT) with this activity being inhibited by *wt* AdE1A.[38,39,52] EMT is accompanied by the breakdown of intercellular junctions which is seen as a reduction in β-catenin, E-cadherin and P-cadherin expression. The increase in β actin expression probably represents a redistribution (and increase in level) from stress fibers to cortical actin. Fibronectin is a well-characterized mesenchymal marker and its expression supports the contention that the 6f transformants are mesenchymal. However, vimentin can be seen at high level in both the AdE1A and 6fE1A expressing cells. Reasons for this are not clear although it has been suggested that neuronal cells are a primary target for AdE1 transformation[55] and if the cells shown here were of neuronal origin this could account for inconsistencies in protein expression. Interestingly there is a notable increase in expression of p21 in Ad126f transformed cells (Fig. 6). This is in contradiction to data obtained with CtBP knock-out cells where p21 is generally up-regulated.[52] It might be expected that neutralization of CtBP, for example by AdE1A, would result in p21 over-expression but in this case the opposite appears to occur. Anomalous results, such as this, have also been seen with other cell lines (eg MCF7) where irradiation causes down-regulation rather that up-regulation of p21 expression (our unpublished data). It is apparent that regulation of p21 expression is complex, particularly in tumour cell lines where it seems that many relevant pathways are de-regulated.

The Effect of siRNA Knock-Down of CtBP Expression on Adenovirus Infection

In an attempt to examine the relative importance of CtBP binding to adenovirus E1A during infection an alternative approach was also adopted. SiRNAs complementary to CtBP1 and CtBP2 mRNA sequences were used to reduce expression of CtBP in A549 cells. These cells, and control cells treated with a random oligonucleotide, were infected with Ad5 *wt* virus. It was observed that knockdown of CtBP1 and CtBP2 facilitated viral infection such that AdE1A and AdE1B19K were expressed to a higher level and at earlier times. Similarly, viral structural proteins, such as penton and fiber, were also expressed at a somewhat earlier time and to a slightly higher level when CtBP proteins were less abundant (Fig. 7). The further addition of siRNAs complementary to CtIP as well as CtBP1 and CtBP2 resulted in an enhanced effect such that there was a greater difference between Ad5 infection of A549 cells in the presence and absence of siRNAs (Fig. 8A and B). Reasons for this are not clear at present as knock-down of CtIP alone had little or no effect on adenovirus infection (data not shown). However these data generally support previous observations, and those presented above, that CtBP acts as a repressor of transcription and that expression of the CtBP binding region of AdE1A results in de-repression.[14] The corollary of this is that AdE1A can regulate transcription of a large number of genes through its action on CtBP. It appears that CtBP has the ability to

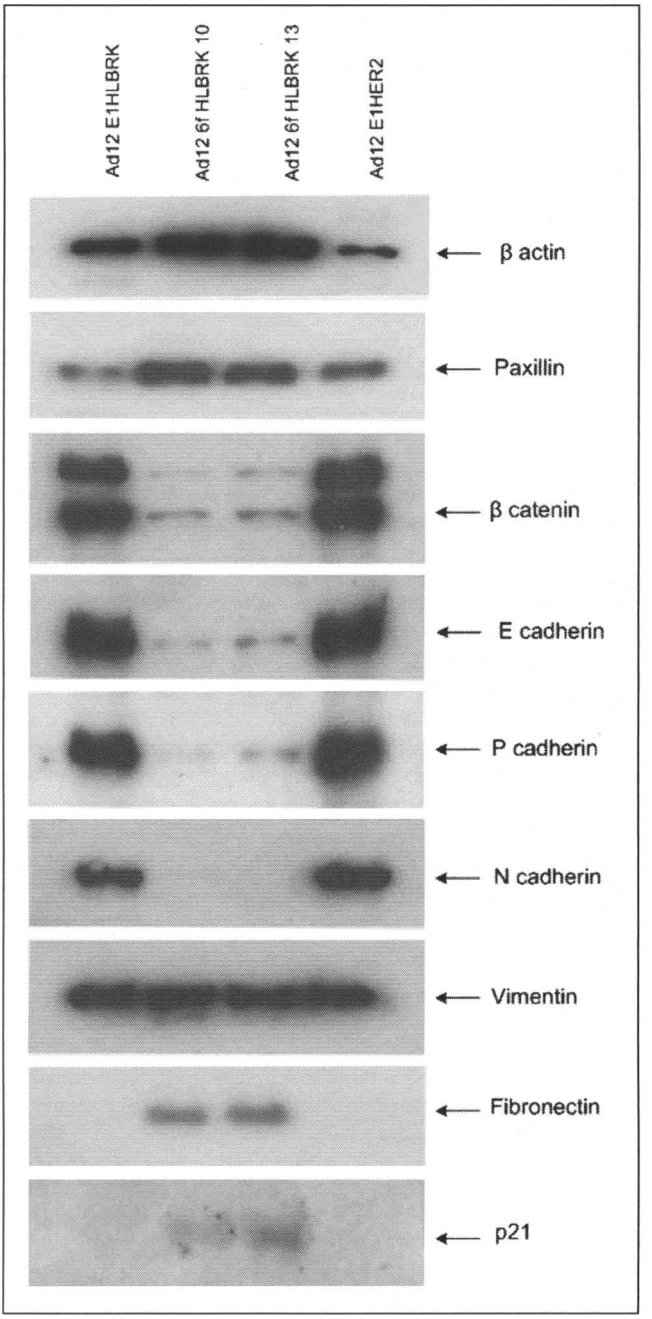


Figure 6. Expression of Ad12E1A causes cells to become more epithelial in nature whereas Ad126f E1A – expressing cells tend to favour a more mesenchymal morphology. Ad12E1HLBRK, Ad126f HLBRK10, Ad126fHLBRK13 and Ad12E1HER2 cell lysates (30 μg of total protein) were fractionated by SDS.PAGE and subjected to western blotting using the antibodies shown.

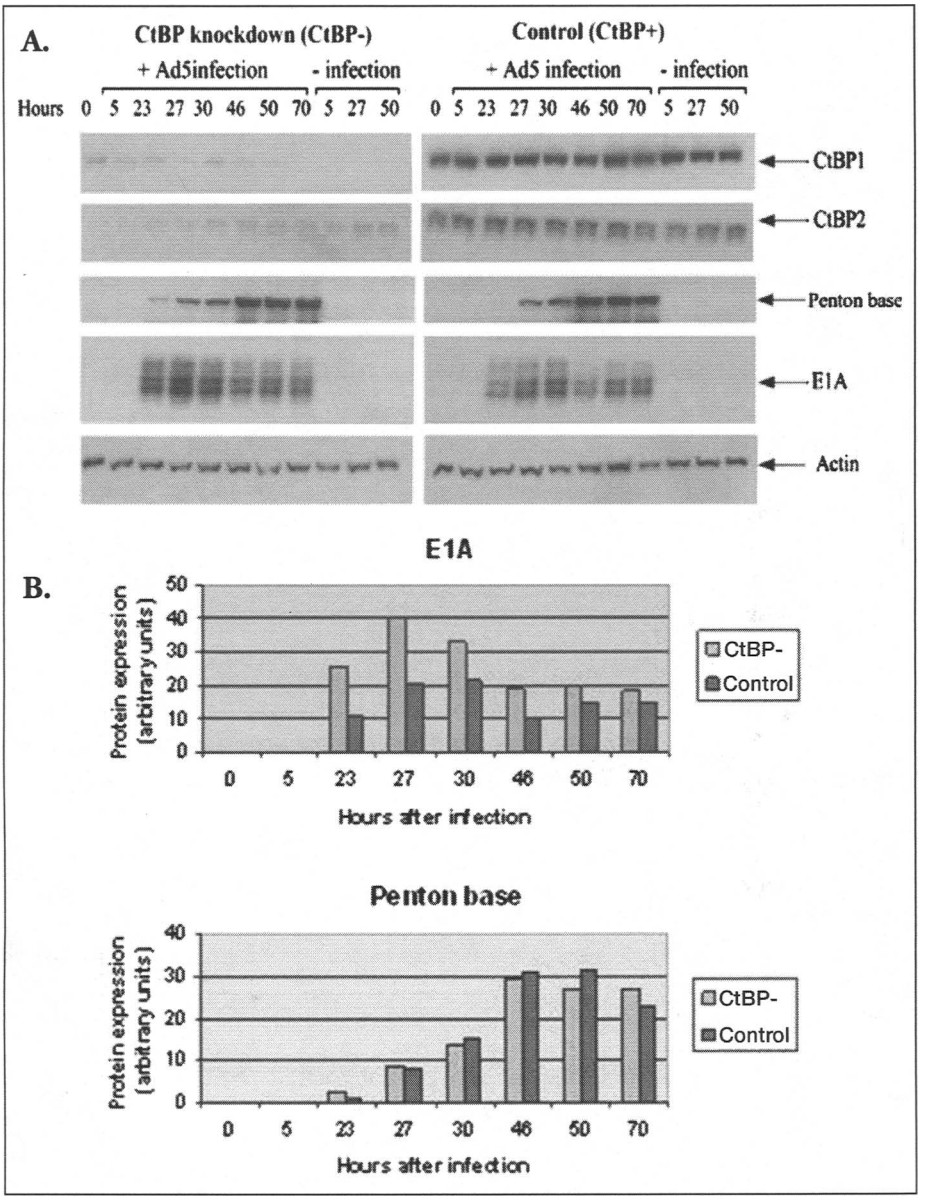


Figure 7. Reduction in host cell CtBP expression facilitates Ad5 infection. A549 cells were transfected with CtBP1 and CtBP2 or control siRNAs. After 3 days cells were infected with Ad5 virus (20pfu/cell). At appropriate times (shown above the tracks) cells were harvested, lysed and subjected to western blotting using the antibodies shown in panel A. The block on the left shows A549 cells infected with Ad5 after knock-down of CtBP1 and CtBP2. The block on the right shows A549 cells infected with Ad5 but after transfection with control siRNA. The three most right-hand tracks in each panel show uninfected cells but after addition of siRNAs. Panel B) densitometric scans of blots shown in A, showing expression of Ad5E1A and penton base.

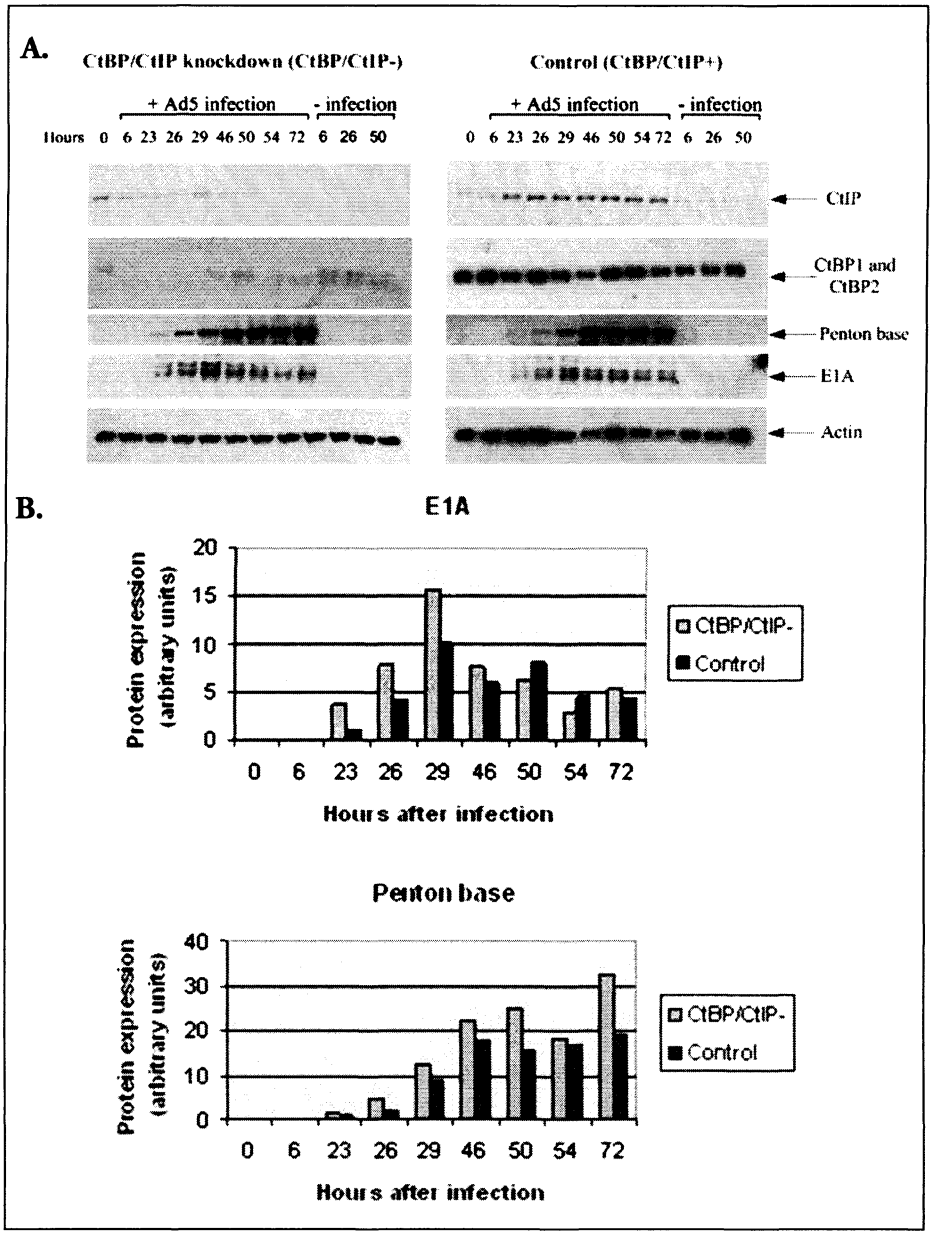


Figure 8. Reduction of host cell CtBP and CtIP expression further facilitates Ad5 infection. A549 cells were transfected with CtBP1, CtBP2 and CtIP or control siRNAs. After 3 days cells were infected with Ad5 virus (20 pfu/cell). At appropriate times (shown above the tracks) cells were harvested, lysed and subjected to western blotting using the antibodies shown in panel A. The block on the left shows A549 cells infected with Ad5 after knock-down of CtBP1, CtBP2 and CtIP. The block on the right shows A549 cells infected with Ad5 but after transfection with control siRNA. The three most right hand tracks in each panel show uninfected cells but after addition of siRNAs. Panel B) densitometric scanning of the western blots shown in Figure 8A showing expression of Ad5 E1A and penton base.

influence the expression of Ad early genes during viral infection and when AdE1A is unable to counteract this by direct binding viral infection proceeds very slowly (Fig. 3D).

Conclusions

The data presented here helps to confirm the importance of CtBP binding to the activity of AdE1A both during viral infection and transformation of rodent cells in culture.[25] A reduction in E1A's ability to bind, and presumably inactivate, CtBP slows viral infection appreciably and has severe consequences for the expression of other early region proteins like E1B54K (Fig. 3). Simplistically it seems that CtBP represses certain transcriptional events necessary for expression of viral genes. Interaction of AdE1A through the PXDLS motif (or loss of CtBP as a result of the action of siRNA oligonucleotides) causes de-repression.[56]

In the original transformation experiments described here it was observed that, at 33°C, 6f E1A was able to transform rat cells in a manner comparable to wild type although with somewhat reduced efficiency (Fig. 1). Presumably at this lower temperature the SVDLS motif adopts a conformation similar to *wt* (PVDLS) and is able to interact with CtBP to a certain limited extent. At higher temperatures transformation is reduced with fewer "epithelial" transformants and many more senescent foci probably because the conformation of Ad12E1A is modified such that it can no longer bind CtBP. It seems that the action of mutant 6f AdE1A is then sufficient to cause the transformed foci to senesce. The activity of the Ad12E1B19K protein is not sufficient to stabilize these colonies. It might be expected that the E1B protein, together with CtBP which has been suggested to possess some anti-apoptotic properties,[52] might allow these foci to develop – clearly this is not the case (Fig. 1) perhaps because it has little or no "anti-senescence" properties. It appears, therefore, that the combined action of AdE1A, which cannot bind CtBP and free CtBP itself is sufficient to cause limited transformation but these cells are unstable and die over the course of a few weeks. As noted in the results presented above it is impossible to confirm temperature dependent differences in the ability of 6f E1A to bind CtBP because of the great difficulty of maintaining samples at such closely regulated temperatures during the course of the pull-down or coimmunoprecipitation experiment.

It has been known for a considerable time that the effect of CtBP binding to AdE1A is quite different in transformation experiments performed with Ad5E1A and AdE1B and with Ad5E1A and mutant *ras*.[14,30,44,45] In the former case, as has been reported here, loss of CtBP interaction capability will reduce transformation appreciably whilst in the latter it produces "supertransforming" E1A. The obvious conclusion to be drawn is that in the case of E1A + *ras* transformation CtBP provides an activity which potentiates transformation and this is inhibited by AdE1A binding. In the case of AdE1- mediated transformation CtBP primarily exerts a repressive effect which is neutralized by E1A interaction. Further studies have shown that CtBP binding also negatively regulates oncogenesis in the Ad5E1A + *ras* model.[14] BRKs transformed with *wt* Ad5E1A and *ras* are not tumourigenic in the syngeneic host whereas cells expressing Ad5E1A with a deletion in the CtBP binding site and *ras* were highly tumourigenic. In the case of Ad12, however, it should be remembered that Ad12E1A - only transformants are tumourigenic although with a long latent period. Ad12E1A + E1B transformants are highly tumourigenic.[48] Whether Ad12E1A + *ras* transformed rat cells cause tumours even more rapidly than Ad12E1 remains to be seen.

The differential regulation of expression of the two major E1B proteins by the 6f mutant E1A is difficult to explain. In the established cell lines E1B19K protein is seen at similar levels to the AdE1 transformants (Fig. 2) but E1B54K protein appears to be totally absent – this suggests that different factors are involved in regulation of expression of the two E1B proteins with CtBP playing a part in one of them but not the other. During infection with the 6f virus E1B19K protein could be detected at levels comparable to AdE1A (data not shown) but E1B54K could only be seen at very late times (Fig. 3), consistent with the results obtained with the transformed cells.

There is considerable evidence to support the suggestion that AdE1A expression in transformed or tumour cells favours expression of an epithelial phenotype through its interaction with, and inhibition of, CtBP.[38-40,52] Thus E cadherin, β catenin and P cadherin are upregulated whereas proteins which are normally expressed in mesenchymal cells, such as vimentin and fibronectin, are absent. The data shown in Figure 6 are generally consistent with this thesis, in that when AdE1A is unable to bind CtBP (in the 6f mutation) cells tend to the mesenchymal phenotype and there is reduced expression of, for example, E cadherin and β catenin. The expression of vimentin in all of the cells, even in the presence of AdE1A, is somewhat difficult to understand and will require further investigation.

The results presented here, together with previous studies, clearly indicate that the presence of "free" CtBP in the host cell has an inhibitory effect on adenovirus infection and transformation.[14,30,35,41] In a complementary series of experiments the effect of CtBP knock-down on Ad infection was examined. It was found that reduction in CtBP expression increased the overall level of viral protein expression as well as reducing the times at which protein expression was first observed (Fig. 7). This was particularly marked when CtIP was also reduced (Fig. 8). As the addition of CtIP siRNA alone had no detectable effect on viral infection (data not shown) it is possible that the inhibition of an activity attributable to the CtIP/CtBP complex may facilitate infection as well as loss of CtBP's transcriptional repressive properties.

Acknowledgements

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CHAPTER 6

CtBP Proteins in Vertebrate Development

Jeffrey D. Hildebrand*

Abstract

The fundamental question facing developmental biology is how the diversity of cell and tissue types that comprise a vertebrate organism can be generated from a single fertilized egg. A critical aspect of the developmental process is setting up and maintaining the differential gene expression that is required to establish the variety of cell lineages present in the adult organism. Thus, an important aspect of understanding development is understanding how early asymmetries in the transcriptome of various cell types are established and, once established, how they are maintained or modified through subsequent generations and differentiation events. This process is carried out by the combined activities of both sequence specific DNA binding factors and their associated coactivators and corepressors that act on either the general transcriptional machinery or the histone component of chromatin. CtBP proteins comprise one branch of corepressors that get recruited to DNA via sequence-specific DNA binding proteins and regulate gene expression. In mice, the CtBP family proteins are encoded by two loci, *Ctbp1* and *Ctbp2*. The transcripts encoding the CtBP1 and CtBP2 proteins are widely expressed and exhibit both unique and shared expression domains in the developing embryo. Genetic analysis of mice harboring mutations in *Ctbp1* and *Ctbp2* indicate that the proteins they encode likely have redundant functions during embryogenesis but are differentially required for specific developmental processes. This analysis shows that CtBP proteins are important in the formation of the placenta and tissues derived from all three germ layers, including muscles, skin, neural ectoderm, and intestinal epithelium. This chapter focuses on the roles of CtBP1 and CtBP2 proteins in vertebrate development, with an emphasis on the genetics of *Ctbp1* and *Ctbp2*, the possible pathways that utilize CtBP proteins during embryogenesis, and the evidence that CtBP proteins could be implicated in multiple developmental processes linked to human diseases.

CtBP Expression in Vertebrate Development

In vertebrates, the *Ctbp* gene family is likely comprised of two loci, *Ctbp1* and *Ctbp2*, which appear to encode at least 4 protein isoforms. The *Ctbp2* locus encodes CtBP2 and Ribeye, a protein isoform with a different N-terminus that is encoded from the alternative inclusion of exons 5' to the start site of *Ctbp2*.[1,2] The *Ctbp1* locus appears to encode CtBP1 and CtBP3/BARS, again via differential expression of 5' exons.[3] In both mice and *Xenopus*, CtBP genes exhibit widespread expression patterns and, between the two genes, are likely expressed in all cell types of the developing embryo.[4-6] This is similar to what is seen in *Drosophila*, where *dCtbp* is maternally expressed and deposited in the embryo and is uniformly expressed in the embryo, albeit at lower levels, following the onset of zygotic gene

*Jeffrey D. Hildebrand—University of Pittsburgh, Department of Biological Sciences, 247 Crawford Hall, Pittsburgh, Pennsylvania 15260, U.S.A. Email: jeffh@pitt.edu

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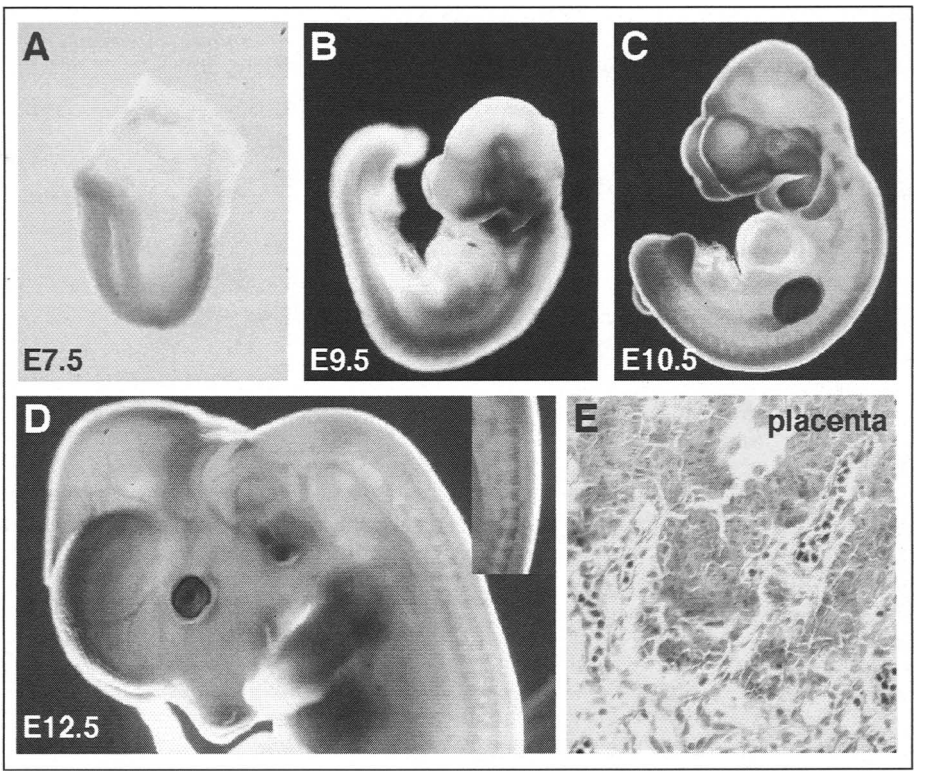

Figure 1. Expression of Ctbp2 during mouse development. A-D). Mouse embryos were isolated at the indicated developmental stages and stained to detect the activity of the *Ctbp2* promoter by staining embryos with X-gal to detect expression of the β-galactosidase reporter in the gene trap cassette integrated into the *Ctbp2* locus. In (A), anterior is to the left. Inset in (D) shows expression in the dorsal root ganglia. E) The placenta from an E.10.5 embryo heterozygous for the gene trap insertion into *Ctbp2* was isolated, stained with x-gal, and sectioned to demonstrate expression in the chorio-allantoic plate (cp) and vasculature of the labyrinth layer (lb).

expression.[7,8] Furthermore, the expression of *Ctbp2* is essentially uniform in the embryo at embryonic day (E) 7.0 and appears to be more dynamic as development progresses (Fig. 1). While the expression of *Ctbp1* and *Ctbp2* appear to be widespread, there are clear regions where these genes are more highly expressed. From approximately E7.0-E10.5 of development, *Ctbp2* expression is essentially uniform. However, by E12.5, regions of higher expression are seen in the developing nervous system, eye, and ear. In extraembryonic portions of the embryo, *Ctbp2* expression is restricted to the vasculature of the yolk sac, the chorio-allantoic plate, and the vasculature of the labyrinth layer of the placenta. This expression is consistent with the defects observed in *Ctbp2* mutant embryos (see below).

Like *Ctbp2, Ctbp1* is also widely expressed in the developing mouse embryo and shows highest expression in the neural epithelium.[4,5] The largely overlapping expression of these two genes in the embryo likely accounts for the lack of embryonic phenotypes observed in the individual knock-outs lines of mice. Consistent with this idea, is the observation that there is little detectable CtBP1 protein in the extra-embryonic structures that express CtBP2.

Redundant and Nonredundant Roles of CtBP1 and CtBP2 in Development

The functions of CtBP1 and CtBP2 have been assessed using a combination of gene targeting and retroviral mutagenesis in ES cells.[4] Mice homozygous null for a targeted mutation in the *Ctbp1* locus is viable but exhibits reduced fitness and fertility. In contract, mouse embryos homozygous for a retroviral gene trap insertion in *Ctbp2* die between E9 and E10.5 of catastrophic defects in numerous tissues structures and there can be quite differing severity of the phenotype. An example of the different observed phenotypes is shown in Figure 1. However, regardless if embryos display either the mild or severe phenotype, lethality appears due to an underlying defect in formation of the placenta. CtBP2 deficient animals fail to form the correct placental architecture, such that there is aberrant morphogenesis of the labyrinth layer. This lesion appears to stem from the inability of *Ctbp2* null cells to form the invasive vascular system needed to elaborate the formation of this structure. Unfortunately, the defects in placenta formation complicate the interpretation and limit the ability to study later roles for CtBP2 in embryonic and adult life. However, in addition to this underlying cause to death, *Ctbp2* null embryos display defects that are likely primary and not secondary to the failure in placental defects, including posterior truncation of the body axis and malformation of the neural ectoderm.[4] Consistent with this defect is the loss or reduction of the expression of *brachyury*, a T-box class transcription factor required for axis formation in mice.[9,10]

Characterization of mice and embryos harboring various combinations of *Ctbp1* and *Ctbp2* mutant alleles has proved complicated, but interesting and informative. First, results from these experiments verify the role of CtBP proteins in a variety of developmental pathways, as might be expected based on the large number of transcriptional regulators that bind both CtBP1 and CtBP2 in vitro and in vivo. Second, these experiments definitively show that CtBP1 and CtBP2 are functionally redundant for at least some aspects of their cellular activities. Mice that are heterozygous for *Ctbp1* or *Ctbp2* are viable and fertile and exhibit no long-term deficits. In contrast, mice that are heterozygous for both *Ctbp1* and *Ctbp2* are typically 30% smaller in mass than their littermates and often die before post-natal day (P) 20. However, those compound heterozygous animals that survive to adulthood are fertile and live a relatively normal life span, although they remain smaller and size and are less robust than normal littermates. These mice exhibit no increased incidents of tumors, which is interesting in light of the numerous reports documenting the interaction of CtBP with various tumors suppressor proteins and repressive complexes. As indicated above, *Ctbp1* $^{-}$ mice are viable, but reducing the gene dosage of *Ctbp2* to one (*Ctbp1* $^{-}$; *Ctbp2* $^{+-}$) results in embryonic lethality. These embryos typically die between E15 and E17 of development and exhibit defects in multiple organ systems, including muscle, skin, intestines, lung, and neural development (Fig. 2B,C). Interestingly, many of the defective tissues and organ systems have extensive epithelial components and these appear to be the most drastically affected cell type. This could be of particular importance based on the evidence that the CtBP and ZEB (Sip/deltaEF1) proteins appear to work together to regulated the expression of E-cadherin and other proteins involved in determining or maintaining epithelial character.[11] This complex may be the target of viral and cellular proteins as a mechanism for regulating cell-cell adhesion and epithelial cell behavior, as the viral oncoprotein E1A and the cellular protein Pinin have been shown to bind CtBP, resulting in de-repression of the E-cadherin promoter.[12,13]

Similar to the above set of genetic interactions, reducing the dosage of *Ctbp1* on a *Ctbp2* -null background results in a more severe phenotype. *Ctbp1* $^{+-}$; *Ctbp2* $^{-}$ embryos arrest approximately one day earlier than do *Ctbp2* mutant embryos and exhibit a more severe phenotype. These embryos do not complete the turning process, fail to complete neural tube closure, and have under-developed heart tissue (Fig. 2D). Finally, embryos deficient for both *Ctbp1* and *Ctbp2* exhibit the most severe phenotype, dying at approximately E8.0 due to

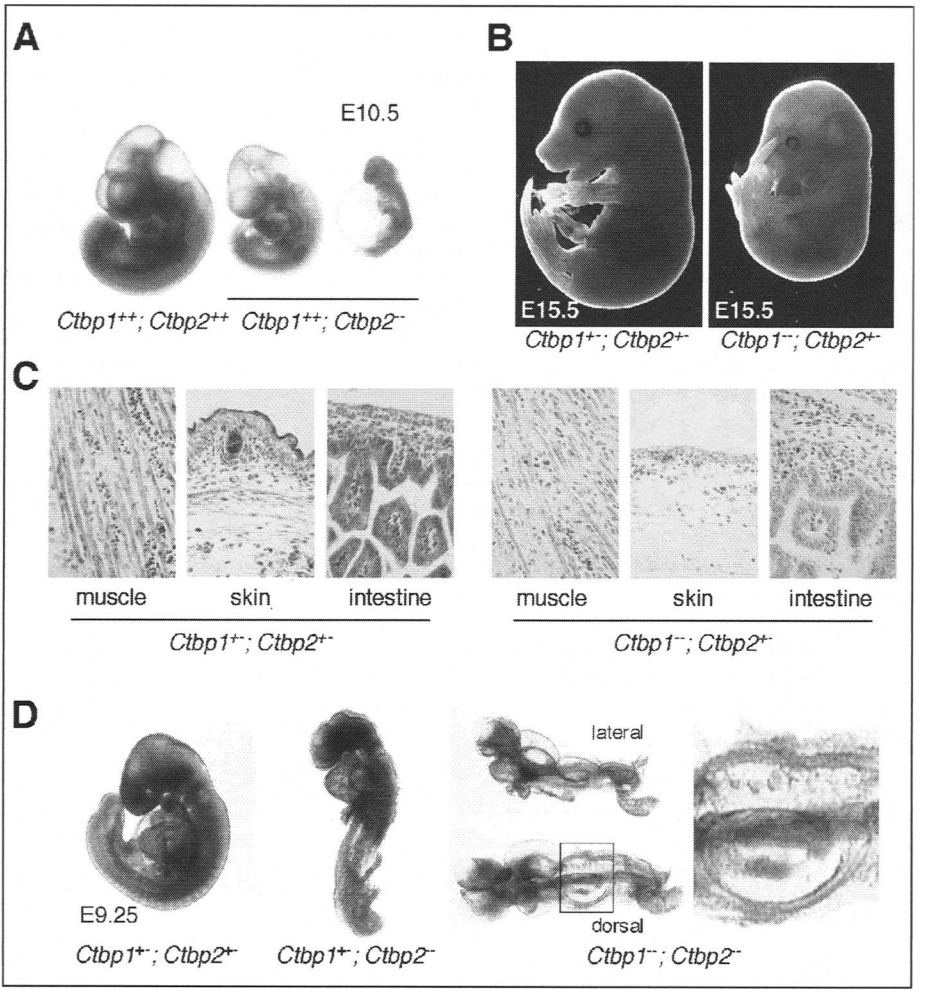

Figure 2. Phenotypes of mouse embryos harboring various combinations of mutant alleles of *Ctbp1* and *Ctbp2*. A) Phenotypes of normal and *Ctbp2* null embryos at E10.5 of development. Although embryos deficient for *Ctbp2* exhibit phenotypes of varying severity, all show decreased size, a dilated pericardium, and reduced growth of the neural epithelium. B,C) *Ctbp1* $^{+-}$; *Ctbp2* $^{+-}$ and *Ctbp1* $^{--}$; *Ctbp2* $^{+-}$ embryos were isolated at E15.5 (B) and evaluated by histology (C). Histological analysis indicates defects in multiple developing organ systems and cell types, including muscle, skin, and intestine. D) Phenotypes of *Ctbp1* $^{+-}$; *Ctbp2* $^{--}$ and *Ctbp1* $^{--}$; *Ctbp2* $^{--}$ embryos isolated at E9.25 of development. Embryos show defects in ectodermal and mesodermal derivatives.

pleiotrophic defects, including aberrant segmentation of somites, heart morphogenesis, and neural development. Two phenotypes that are of particular interest are a lack of segmental patterning of the somatic mesoderm and the extensive blebbing of what is presumably ectoderm (Fig. 2D). The lack of proper segmentation is consistent with the defects seen in *Drosophila* embryo lacking maternal *dCtbp*.[8] The blebbing defects could be the result of early adhesive defects, which is consistent with some of the known targets of CtBP. These defects are probably not secondary to defective extraembryonic development, since the placental

functions do not become established until later in development and the allantois exhibits normal morphology (Fig. 2D) and appears to attach normally to the chorion.[4]

These observations might also shed light on the proposed cytoplasmic functions of CtBP3/BARS.[3,14,15] If this protein is encoded by *Ctbp1* and not a different genomic locus, then the cytoplasmic function of CtBP3 is likely not essential for viability of the early embryo. This is based on two basic principles. First, there do not appear to be any gross defects in golgi structure in the cells null for *Ctbp1* and *Ctbp2*. In these cells, western blot analysis does not detect any CtBP1 or CtBP2 using antibodies to the C-termini of these proteins. Since CtBP1 and CtBP3/BARS are identical in the C-terminus, even if was encoded by a different gene it should still be detected. In addition, the observation that null embryos survive until day E8.0 suggests that there are no dramatic defects in golgi function. Since maternal messages are degraded at the two-cell stage of mouse development and any maternally deposited protein would need to remain functional for eight days, it is unlikely that early requirements for CtBP3/BARS are masked by maternal contributions to the embryo.

Developmental Genetics of CtBP

While the pleiotropic defects observed in CtBP deficient embryos implicate these proteins in many developmental processes, the identities of the pathways that might be compromised in these mutants is unclear. This is mostly due to the fact that CtBP has been reported to bind to a multitude of transcription factors of diverse proposed functions. In addition, few of the known factors that associate with CtBP have been mutated by gene targeting, thus precluding genetic analysis. However, there are some commonalities shared between embryos lacking functional CtBP and embryos harboring mutations in genes that encode known CtBP binding proteins. As outlined above, *Ctbp1*$^{-/-}$; *Ctbp2*$^{+/-}$ embryos show defects in muscle and skeletal differentiation. This is consistent with the association of CtBP with MEF2 complexes and the skeletal defects observed in deltaEF1 null mice, respectively.[16-18]

Perhaps the best indications of which developmental process utilize CtBP functions come from the recent papers showing the involvement of CtBP in both Wnt and TGF-β/BMP signaling pathways.[6,19-25] CtBP proteins have been linked to the TGF/BMP pathway via their association with the BMP receptors (BMPR-II) and the transcription factors, Sip/ZEB/deltaEF, TGIF 1/2, and Evi-1 and to the Wnt pathway via association with APC and TCF. These pathways are involved with essentially every developmental process. The wide range of phenotypes observed in *Ctbp* null animals may stem from the diverse activities of these pathways during vertebrate development. In addition, only a subset of the numerous cellular responses initiated by these pathways may be perturbed in the absence of CtBP function.

The other activity of CtBP that might have a dramatic impact on vertebrate development is the regulation of epithelial cell character. This is particularly important during epithelial-mesenchymal transition (EMT), a term that describes the process by which cells alter their physical characteristics and switch from an epithelial to fibroblastic phenotype (or the converse). The activity of CtBP proteins has been linked to this process in transformed cells via the repression of genes that encode regulators of cell-cell adhesion and epithelial behavior.[11] Breakdown of intercellular adhesion is critical for epithelial cells to adopt the motile behavior associated with the fibroblastic phenotype. Cells and embryos lacking CtBP function show dramatic increase in the expression of E-cadherin.[11] Thus, regulating the activity or expression of CtBP could have important impacts on the physiology of certain cell types. E-cadherin expression is not only important for EMT, but is likely required for normal cell-cell adhesion and tissue homeostasis in several tissues during vertebrate development.

The phenotypes seen in *Ctbp1* and *Ctbp2* -deficient mice suggest that mutations in these genes in humans may result in embryonic lethality. However, the association of CtBP with such a large number of different classes of transcription factors and their likely involvement in numerous developmental processes implies that alterations in either the interaction of CtBP

with specific factors or alterations in the dosages of *Ctbp1* or *Ctbp2* may be sufficient to cause developmental disorders. This could be of particular significant when considering the number of genetic modifiers that might exist for *Ctbp* genes. Perhaps the best example of this model is seen in the case of the transcription factor TGIF and its association with the human syndrome Holoprosencephaly (HPE), a condition resulting from defects in midline patterning of the neural ectoderm and other anterior structures.[26] Afflicted individuals exhibit defects of varying severity and include cyclopia, cleft lip or palate, and mental retardation due to defects in patterning the cerebral cortex. HPE4, one of the loci associated with holoprosencephaly in humans, maps to *TGIF*.[27] Mutations in TGIF include several single amino acid changes in conserved residues in the DNA binding domain and SMAD binding domains. Another allele of TGIF is caused by a point mutation that disrupts that interaction between CtBP and TGIF in vivo.[22] Importantly, the interaction between CtBP and TGIF is critical for establishing the full repressor function of TGIF in response to BMP signaling.[22] Some of the defects observed in *Ctbp* deficient mice are consistent with those presented by patients with HPE. Most *Ctbp2* mutants, as well as *Ctbp1* and *Ctbp2* compound mutants, display defects in the anterior neural tube, including failure of midline closure as well as under growth of the telencephelon in the forebrain.

Conclusions

Elucidating the in vivo functions of CtBP proteins has proved to be quite challenging, particularly when it comes to balancing the dramatically different roles it appears to play in the cytoplasm, the nucleus, and synapses. However, it is clear that these proteins are required for numerous processes during vertebrate development and likely play key roles in human diseases. The next level of research will be to understand the specific spatial and temporal requirements for CtBP proteins during development.

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CHAPTER 7

CtBP as a Redox Sensor in Transcriptional Repression

Qinghong Zhang,* Clark C. Fjeld, Amanda C. Nottke and Richard H. Goodman

Abstract

The corepressor CtBP (carboxyl-terminal binding protein) is involved in transcriptional pathways important for development, cell cycle regulation, and transformation. We demonstrate that CtBP binding to transcription repressors is stimulated by NAD^+ and NADH, with NADH being two to three orders of magnitude more effective. Fluorescence resonance energy transfer studies of CtBP show a >100-fold higher affinity for NADH than NAD^+, in agreement with the tighter interaction observed in the crystal structure of NADH-bound CtBP. Levels of free nuclear nicotinamide adenine dinucleotides, determined using two-photon microscopy, correspond to the concentrations required for half-maximal CtBP binding. Free cellular NAD^+ concentration greatly exceeds that of NADH and the redox changes are mainly reflected by NADH levels. Agents increasing NADH levels stimulate CtBP binding to its partners in vivo and potentiate CtBP-mediated repression. These findings suggest that the transcriptional corepressor CtBP may serve as a redox sensor to provide a link between gene expression and metabolism.

Introduction

The metabolic state of a cell is thought to influence cellular functions including transcription. Recently, a few connections have been made between changes in metabolic state and effects on gene regulation through the action of nicotinamide adenine dinucleotides. The focus of this chapter is the regulation of gene expression by the metabolic state of the cell through the electron carrier redox pair NAD^+/NADH and the ability of the transcriptional corepressor CtBP to serve as a redox sensor. We hypothesize that CtBP provides an important link between gene expression and metabolism.

Differential Binding of CtBP to NAD^+ and NADH

The carboxyl terminal binding protein (CtBP) is a transcriptional corepressor important for development, cell cycle regulation, and transformation.[1] CtBP was first identified through its ability to interact with the carboxyl terminus of adenovirus E1A oncoprotein.[2] The residues in the carboxyl terminus critical for the interaction were determined to be the PLDLS sequence.[3]

*Corresponding Author: Qinghong Zhang—Vollum Institute L-474, Oregon Health and Science University, 3181 S.W. Sam Jackson Park Road, Portland, Oregon 97239, U.S.A. Email: zhangq@ohsu.edu

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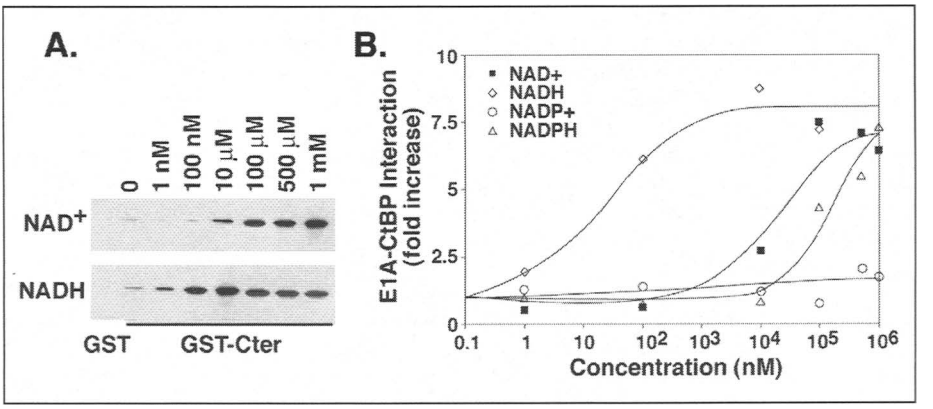

Figure 1. A) Binding of recombinant CtBP to GST-E1A at various concentrations of NAD^+ and NADH. B) Relative interactions as a function of nicotinamide adenine dinucleotide concentration depicted on a log scale.

The dehydrogenase domains of CtBP and 3-phosphoglycerate dehydrogenase are nearly 40% identical. A weak dehydrogenase activity has been reported for CtBP in the presence of pyruvate and NADH,[4-6] but the turnover rate of CtBP is 30,000-fold less than that of lactate dehydrogenase.[7] Furthermore, the enzymatic activity of CtBP, which can be blocked by mutation of the catalytic center His 315, is not required for repression of target genes in CtBP-knockout mouse embryo fibroblasts.[8] Previous transcriptional tethering studies have also shown that the His residue of the catalytic triad is dispensable for the transcriptional repressive activity of mCtBP2[9] and dCtBP.[10] This suggests that the dehydrogenase activity is not essential for CtBP-mediated gene repression.

Nonetheless, it is possible that CtBP, like the dehydrogenases and reductases, is regulated by NAD^+ or NADH in some other capacity. One model that we considered was that NAD^+/NADH could affect the ability of CtBP to interact with its partners. To test this hypothesis, we examined the interaction of bacterially-expressed CtBP with GST-E1A fusion proteins at different concentrations of NAD^+/NADH. To our surprise, CtBP binding was regulated dramatically, with NADH increasing the interaction at concentrations in the nM range (Fig. 1). NAD^+ also increased binding, but was two-to-three orders of magnitude less effective. $NADP^+$ and NADPH had little if any effect (Fig. 1).

Because NAD^+/NADH similarly affected CtBP binding to a variety of transcriptional repressors, we speculated that the nicotinamide adenine dinucleotides functioned by altering CtBP structure. Support for this idea was obtained from limited proteolysis assays demonstrating an induced conformational change of CtBP upon NAD^+/NADH binding.[11] This observation agrees with numerous studies of dehydrogenase structure and function. In general, nucleotide binding to dehydrogenase proteins induces a conformational change involving the movement of a flexible loop over the active site, thus favoring catalysis by optimizing the positions of catalytic and substrate binding residues. NADH protects CtBP from the proteolytic digestion at a lower concentration than NAD^+.[11]

Regulation of CtBP binding to its partner by NAD^+/NADH has attracted much attention and is somewhat controversial. Although the ability of nicotinamide adenine dinucleotides to stimulate CtBP binding to E1A and other proteins has been confirmed by several laboratories,[4,5] the differential efficacy of NAD^+ and NADH has been challenged. We showed that NADH was two-to-three orders of magnitude more effective than NAD^+ in stimulating CtBP binding and proposed that this differential effect might link CtBP-mediated repression to the redox state of the nuclear compartment. In contrast, Kumar et al[4] and Balasubramanian et al[5]

concluded that NAD^+ and NADH were equally effective in stimulating CtBP binding. While Balasubramanian et al did not examine levels of NAD^+ and NADH that were low enough to discern a differential effect, Kumar et al tested a larger range of nicotinamide adenine dinucleotide concentrations and claimed that the single proton difference between NAD^+ and NADH could not result in a two-to-three order of magnitude difference in binding.

We suspect that the disparate results of various laboratories on the differential binding of CtBP to NAD^+ and NADH reflects the fact that NADH binds tightly and cannot easily be removed. Kumar et al synthesized ^{35}S-labeled CtBP by in vitro transcription/translation for use in the GST-pull down assays. This approach allowed them measure the CtBP/E1A interaction by detecting CtBP binding using autoradiography and avoids difficulties associated with quantification using Western blot techniques. However, it is possible that the CtBP protein synthesized in vitro is already associated with NADH. Additionally, the lowest concentration of NADH tested was 10-fold higher than the EC_{50} (100 nM) that we reported. Balasubramanian et al used purified CtBP expressed in bacteria and analyzed the levels of CtBP that interacted with GST-E1A by Western blotting. The lowest concentration of NADH used was 10 μM. A challenge associated with measuring high affinity binding events is to determine how much protein should be used in the binding assay. This level used must be high enough to obtain a good signal/noise ratio. However, higher levels of protein require more substrate to achieve saturation. If the EC_{50} is in the low nanomolar range and the protein used is in the micromolar range, the true binding value will not be observed as the amount of substrate required to saturate all the protein-binding sites depends on the protein concentration. It is possible that the more sensitive methods used in our study may have allowed the differential effects of NAD^+ and NADH on the CtBP-E1A interaction to be observed more clearly.

Because the potential for CtBP to serve as a redox sensor depends upon its differential affinity for NAD^+ and NADH, we decided to investigate these binding events directly. Structural studies showed that Trp 318 of CtBP lies within four angstroms of the nicotinamide in NADH. Thus, we predicted that we could directly measure the binding of NADH by measuring the transfer of energy from the adjacent tryptophan. The binding of NADH to protein is associated with an enhanced intensity and blue-shift of NADH fluorescence.[12] NAD^+ affinity was estimated by measuring the loss of the fluorescence blue shift, as NADH dissociates upon addition of NAD^+. Our studies showed that CtBP has a greater than 100-fold higher affinity for NADH than NAD^+, consistent with the proposed function of CtBP as a nuclear redox sensor. Interestingly, the affinities of NADH and NAD^+ for CtBP are very reminiscent of those determined for 3-phosphoglycerate dehydrogenase over 40 years ago.[13] Because NAD^+ and NADH appear to cause similar changes in CtBP binding, albeit at different concentrations, it will be important to determine what fraction of CtBP is occupied by these ligands in vivo.

To better understand the molecular mechanism underlying the differential binding of NAD^+ and NADH to CtBP, we analyzed the crystal structure of CtBP bound to the reduced and oxidized form of the nucleotide.[4,14] The structural data reveal that CtBP takes advantage of the positive charge on NAD^+ to distinguish it from NADH. The lack of repulsion between charged residues and NADH favors CtBP binding. In addition, the nicotinamide carboxamide is rotated to a staggered position when CtBP is bound to NADH, while it is planar in the structure of CtBP bound to NAD^+. The distances between CtBP residues and NAD^+ and NADH are also different: Arg 266 and His 315 are 4.18 and 6.24 angstroms from N1 of the nicotinamide ring of NAD^+, respectively. In the structure with bound NADH, these distances are reduced to 3.69 and 5.60 angstroms (Fig. 2), supporting the idea that NADH binds more tightly to CtBP. The result of the carboxamide rotation appears to be stronger hydrogen bonds between CtBP and N7 of the amide based on the shorter interatomic distances. These findings are consistent with the idea that CtBP prefers NADH over NAD^+.

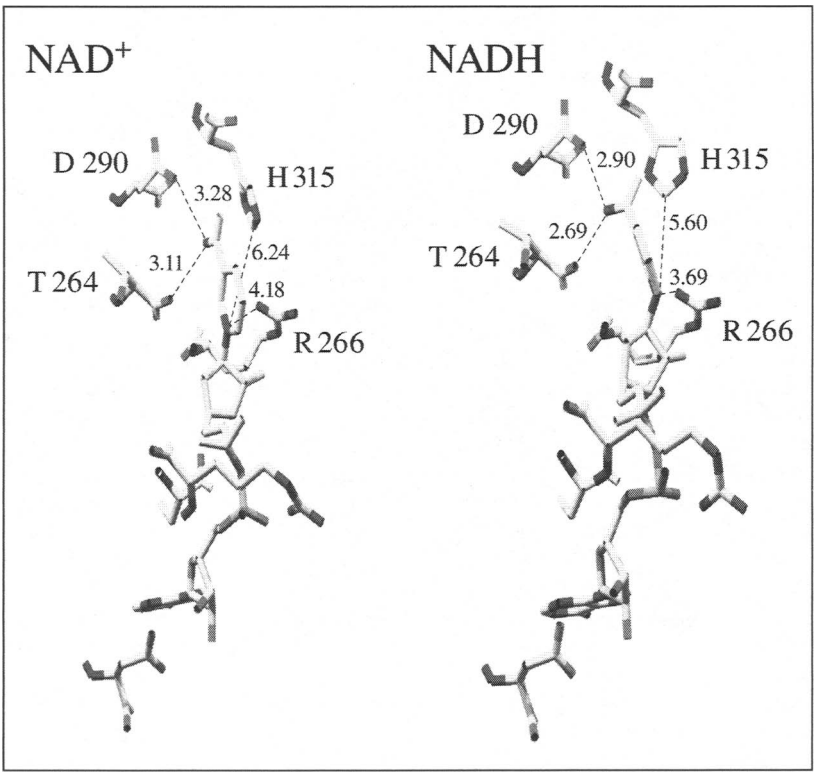

Figure 2. Structural data suggesting preference of CtBP for NADH over NAD^+, taken from references 14 and 4, respectively. Contacts demonstrating higher affinity for the reduced nicotinamide are shown. These structures were taken from the Protein Data Bank (accession numbers for CtBP/NAD^+, 1MX3, and CtBP/NADH, 1HL3).

NADH as Redox Indicator

The metabolic state of a cell encompasses the energy state and the redox state. The energy state describes the availability of fuel sources (glucose, lipids) and useable energy (ATP) while the redox state describes the oxidative potential that reflects the balance of the molecules able to undergo oxidation/reduction reactions. NAD^+ and NADH reflect both the energy state and oxidative state of a cell. NADH is a product of glycolysis and the TCA cycle. The breakdown of carbon sources is associated with the reduction of NAD^+ to NADH. NADH is also a substrate for oxidative phosphorylation, where its reducing equivalents are transferred to molecular oxygen to yield water and provide the proton gradient required for ATP synthesis. The integral role of NADH in ATP synthesis, as a product of carbon breakdown and a substrate required for maintaining a proton gradient, demonstrates the ability of NADH to serve as an indicator of the cellular energy state. NADH is also in equilibrium with NADPH. The NADPH-dependent reduction of glutathione is critical for maintaining protein cysteine residues in a reduced state. NADH transhydrogenase monitors the balance between NADH and NADPH levels. Thus, NADH levels indirectly reflect levels of molecular oxygen and other oxygen species related to oxidative stress such as hydrogen peroxide, superoxide, hydoxyl radical, and nitrogen oxide. The function of NADPH in the reduction of glutathione provides the link between NADH and the cellular oxidative state.

The redox pair, NAD^+/NADH, tightly couples to the energy state by serving as the link between fuel sources and useable energy. We hypothesize that the dependence of CtBP on NAD^+ and NADH might allow the transcriptional corepressor to sense both the energy and redox state of the cell. For CtBP to be an effective sensor of cellular energy and redox state, it must be sensitive to changes in the levels of free nucleotides. Our in vitro studies indicate that NADH stimulated E1A binding to CtBP at a level much lower than NAD^+ (i.e., NADH has an affinity for CtBP of approximately 66 nM[12]). The physiological relevance of these observations depends on the concentration of these pyridine dinucleotides in cells. The majority of the pyridine dinucleotides is bound by proteins and unavailable to regulate CtBP. Furthermore, different cellular compartments, such as mitochondria, may contain different levels of pyridine dinucleotides. Thus, it is important to know the concentrations of free NAD^+ and NADH in the nucleus. To address this, we determined the free nuclear NADH level using two-photon microscopy. The two-photon approach allows excitation at a relatively long wavelength, which avoids damage to cellular structures. This technique is thus ideally designed to determine the concentration of NAD(P)H in distinct cellular compartments.[15] Quantitation of the fraction of nuclear NADH that is not bound to protein can be determined using fluorescence lifetime measurements. A value of 130 nM was determined for free nuclear NADH.[11]

Total NAD^+ and NADH nucleotides do not reflect the redox state of free nucleotides in the cytoplasm or the nucleus. Only the free pool of nicotinamide adenine dinucleotides in the nucleus is relevant in controlling transcription. The free cytosolic NAD^+/NADH ratio can be calculated from the substrates of the lactate dehydrogenase reaction and was determined to be 670.[11,16] Because unbound nicotinamide adenine dinucleotide should pass freely through nuclear pores, this difference in the levels of free NAD^+ and NADH should pertain to the nucleus as well. Given that the free nuclear NADH is 130 nM, the concentration of free NAD^+ was then calculated to be about 90 μM.[11] These values are well within the range of free pyridine dinucleotide concentrations in the literature.[17]

The free nuclear NAD^+ level greatly exceeds that of NADH. Given the ratio of free NAD^+/NADH, conversion of NAD^+ to NADH causes a much larger relative change in the NADH level. This implies that the levels of free NADH are likely to more accurately reflect metabolic events than the levels of NAD^+.

NADH-Dependent CtBP Binding to Repressors in Transcription Repression

The dinucleotide binding activity and the dehydrogenase activity seems to be unique to animal CtBPs.[18,19] We hypothesize that CtBP evolved from the dehydrogenases and reductases in a manner that resulted in the loss of enzymatic activity but retention of the capacity to be regulated by NAD^+/NADH. The relative concentrations of free NAD^+ versus NADH suggest that interconversion of the two forms via oxidation/reduction reactions significantly alter only the NADH component. Thus, the association of CtBP with its partners containing PXDLX motifs could be regulated by perturbations in cellular redox state. We found that the free cytoplasmic NAD^+/NADH ratio was decreased in cells treated with $CoCl_2$, azide, or hypoxia, indicating an elevated free NADH level. These treatments significantly and specifically increased CtBP interaction with E1A or ZEB.[11] In contrast, the association between Pak1 and CtBP was not affected by NADH,[20] while the binding of HDM2 to CtBP was down-regulated by treatments that decreased NAD^+/NADH ratios.[21] This suggests that CtBP interacts with its various partners through distinct motifs.

Balasubramanian et al[5] found that NAD(H) doubled the apparent molecular mass of CtBP, suggesting that its addition promoted dimerization. The crystal structures of CtBP are nucleotide-bound and also reveal CtBP dimers,[14] supporting reports by several other groups. Thus, CtBP is able to dimerize in a manner that appears to depend on nucleotide binding. It is possible, therefore, that this ability of NAD(H) to stimulate CtBP dimerization could contribute to the enhanced binding of transcriptional repressors.

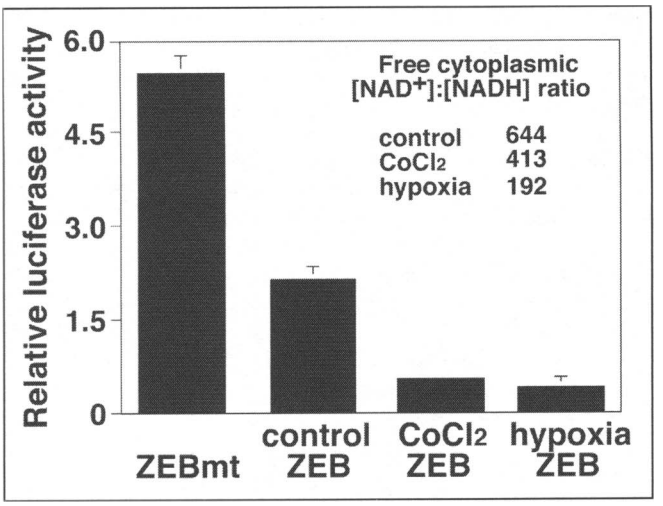

Figure 3. Redox state regulates CtBP interaction in vivo. Hypoxia and $CoCl_2$ enhance CtBP-mediated repression. Cos7 cells were cotransfected with an E-cadherin reporter gene and truncated ZEB constructs containing an E-box binding domain and wild-type or mutated CtBP-binding motifs. Cells were treated with 200 μM $CoCl_2$ or exposed to hypoxia for 16 hours. Inset shows the effects of various treatments on the free cytoplasmic NAD^+/NADH ratio.

The NADH-modulated interaction between CtBP and DNA-binding repressors suggests that CtBP can sense metabolic events altering NADH levels and can translate changes in redox state into transcriptional output. To test whether cellular redox state affects CtBP-mediated repression, we assayed the repression of E-cadherin reporter by ZEB, a prototypical cellular CtBP-binding repressor. ZEB repressed the E-cadherin promoter in a manner that depended on the CtBP interaction sites (Fig. 3). Treatment with $CoCl_2$ or hypoxia significantly enhanced the level of CtBP-mediated repression.

To directly assess the role of NADH in stimulating CtBP binding to transcription repressors, we performed chromatin immunoprecipitation assays in human colon cancer HT29 cells using a CtBP antibody (Fig. 4). The recruitment of CtBP to the E-cadherin promoter was significantly enhanced by $CoCl_2$ treatment that results in NADH increase, further supporting our hypothesis that CtBP serves as a redox sensor for transcription.

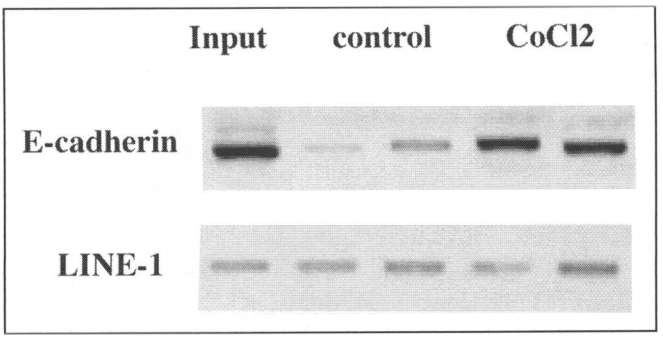

Figure 4. Redox state regulates CtBP recruitment to E-cadherin promoter. HT29 cells were treated with 200 μM $CoCl_2$ for 1 hour before the chromatin immunoprecipitation assay using a CtBP antibody. LINE-1 serves as a control.

Biological Events Associated with NADH Level Fluctuation

There are several events that invoke significant changes in cellular NADH levels. Excessive alcohol consumption initiates a dramatic increase in NADH in the liver as a result of the enzymatic conversion by alcohol dehydrogenase.[22] Large changes in cellular redox state also occur in certain metabolic abnormalities such as diabetes.[16] Several studies suggest a role of elevated NADH levels in diabetes. For example, disruption of the electron transport chain in the murine pancreatic B HC9 cells resulted in elevated NADH levels in the mitochondria and the cytosol leading to suppression of glucose-stimulated insulin release.[23]

In cancer, poor vascularization of tumors results in hypoxia. Because molecular oxygen is the final acceptor of the reducing equivalents carried by NADH, hypoxia elevates NADH levels. The hypoxic state of tumors thus provides an example of how increased NADH levels can affect expression of CtBP target genes and possibly promote certain aspects of malignant transformation. Conceivably, the redox regulation of CtBP function could influence a large number of transcriptional repressor pathways involving CtBP that are operative during these conditions. E-cadherin is probably the best-characterized target promoter for CtBP in mammalian cells[24,25] and loss of E-cadherin expression in both tumor cell lines and primary carcinomas correlates with metastasis, invasion, and poor clinical prognosis.[26,27] The enhanced CtBP repression of the E-cadherin promoter under hypoxic conditions may contribute to tumor invasiveness.

The developing mammalian embryo experiences a hypoxic environment in utero. At birth, a dramatic shift to a hyperoxic environment occurs when the fetus experiences the transition from placental to lung-based respiration. During this transition, the oxygen levels increase from $\leq 3\%$ to 10-15% O_2. Reactive oxygen species generated during this transition influence the oxidative state of cells and serve as signaling molecules. Changes in oxygen levels, and thus the oxidative state, contribute to the environmental cues important for regulating gene expression patterns. Concomitantly, the NAD^+/NADH ratio increases 15-fold at birth,[28] suggesting a possible role of the redox sensor CtBP in preceding developmental events.

Redox Sensitive Nuclear Transcription Regulators

An increasing number of transcription factors have been found to utilize metabolic cofactors, such as nicotinamide adenine dinucleotides, in transcriptional regulation. NAPS2, the mammalian functional analog of *Drosophila* Clock, is a transcription factor regulating the expression of genes involved in circadian rhythms as a function of the light-dark cycle. NPAS2 binds DNA as a dimer with BMAL1, a bHLH-PAS domain-containing protein. Rutter et al[29] demonstrated that NADH and NADPH were able to stimulate the DNA binding of NPAS2:BMAL1 with EC_{50} values of 6.3 and 2.3 mM, respectively. They proposed that the millimolar levels of reduced nucleotides is within the physiological range based on an early study designed to measure total cellular reduced nucleotide levels.[30] Interestingly, they observed that the oxidized form of each nucleotide inhibited DNA binding. The IC_{50} of $NADP^+$ on NPAS2:BMAL1 DNA binding was reported to be 0.56 mM. Further DNA binding studies revealed that the ratio of reduced to oxidized nucleotide had a significant impact on the DNA binding ability of NPAS2:BMAL1. They concluded that NPAS2 and Clock proteins serve as redox sensors with the ability to sense changes in the NADPH/$NADP^+$ and NADH/NAD^+ levels. The ratios observed to elicit the greatest response were between 60:40 and 80:20 (reduced to oxidized) dinucleotides. How these observations relate to the DNA binding of these proteins in vivo is unclear, however, given that the free NAD^+ concentration is thought to be several orders of magnitude higher than the free NADH concentration. The free NADH/NAD^+ ratio of 1:700 observed by several laboratories is significantly different than the ratios proposed to regulate NPAS2. Additionally, effects observed at millimolar levels are unlikely to be physiological when the concentration of free NAD^+ and NADH are about 70 μM and 100 nM, respectively.

In addition to regulating DNA-binding, nicotinamide adenine dinucleotides can also influence transcription by regulating protein-protein interactions. The stimulated binding between

CtBP and repressor proteins was the first such example. Subsequently, it was determined that the interaction between glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Oct-1 was also regulated by nicotinamide adenine dinucleotides.[31] GAPDH was identified as a component of the OCA-S coactivator complex important for S phase-dependent histone H2B transcription. NAD^+ and NADH levels were reported to regulate the binding of GAPDH to Oct-1 in a manner that is stimulated by NAD^+ and inhibited by NADH, both at 100 nM level. Whether OCA-S mediated transcription in vivo is regulated by NAD^+ or NADH was not tested. Another dehydrogenase protein, lactate dehydrogenase, was isolated as components of the OCA-S transcriptional coactivator complex as well. The relevance of lactate its enzymatic activity for gene activation through the OCA-S complex remains to be determined.

Besides its role as an allosteric regulator, nicotinamide adenine dinucleotides can also function as substrates for transcriptional cofactors. Studies of the histone deacetylase Sir2 first uncovered the connection between the nicotimamide adenine dinucleotides and gene regulation. In the case of Sir2, the related nucleotides NADH, $NADP^+$, and NADPH, cannot replace NAD^+ as essential cofactors for enzymatic activity.[32] Sir2 consumes NAD^+ and removes an acetyl group from an acetylated substrate. The finding that Sir2 function depends on NAD^+ has led to a far-reaching series of studies linking nutrition, gene silencing, and longevity in a variety of species. Studies performed by Fulco et al[33] describe the role of Sir2 in regulating muscle gene expression and differentiation. They reported that the free nuclear NAD^+/NADH ratio decreases as muscle cells undergo terminal differentiation. By examining the expression of the MHC gene in myoblasts, they found that the pyruvate treatment increased the NAD^+/NADH ratio and decreased MHC expression. They concluded that activated Sir2 function is responsible for transcriptional regulation because the pyruvate effect can be blocked by nicotinamide, a Sir2 inhibitor. Given that pyruvate treatment influences the NAD^+/NADH ratio and thus mainly changes the NADH level, the precise mechanism of Sir2 activation by pyruvate is currently unknown.

In summary, the observation that NADH stimulates the interaction between CtBP and E1A was the first step towards understanding the function of CtBP as a redox sensor in transcriptional repression. We found that the differential binding of CtBP to NAD^+ and NADH correlates with the free NAD^+ and NADH levels in the nucleus. Furthermore, we demonstrated that the dehydrogenase activity of CtBP is not required for gene repression. Instead, we propose that the ability of CtBP to bind NADH is important for regulating its recruitment to promoter elements and gene repression in response to metabolic changes involving NADH.

Acknowledgements

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CHAPTER 8

CtBP Corepressor Complex - A Multi-Enzyme Machinery that Coordinates Chromatin Modifications

Yu-Jiang Shi and Yang Shi*

Abstract

Recent biochemical and proteomic approach has identified a CtBP super complex consisting of a host of chromatin modifying enzymes. Analysis of this complex has led to the appreciation that enzymes that mediate deacetylation and histone H3 lysine 9 methylation are present in the same biochemical complex, which facilitates coordinated histone modifications important for establishing repressive chromatin. Importantly, studies of this complex also resulted in the finding of the first histone demethylase LSDI, which represses transcription by demethylating histone K4, where methylation is linked to active transcription. It is anticipated that additional important new insights will be gained from further investigation of this unusual transcriptional repression machine.

CtBP is a transcriptional corepressor and is one of the three main transcriptional cofactors that are directly targeted by the viral oncoprotein E1A during oncogenic transformation.[1] To explore mechanisms by which CtBP mediates transcriptional repression, a biochemical approach was taken to isolate proteins that are associated with CtBP. This effort has led to the identification of a CtBP super-complex, consisting of, among others, six potential enzymatic activities.[2] While the exact composition of this super-complex may differ in different cell types, characterization of these enzymatic functions in HeLa cells has already provided significant insight into mechanism of action of CtBP and eukaryotic gene regulation. Below we provide a brief discussion of the enzymatic components of the CtBP complex and our current understanding of their individual as well as coordinated enzymatic actions in transcriptional repression. While other aspects of CtBP are covered in other chapters, this chapter is largely confined to the CtBP super complex.

Six Enzymes/Potential Enzymes in the CtBP Super-Complex

CtBP: A Nuclear Dehydrogenase

CtBP shares significant sequence homology with members of the dehydrogenase family, in particular the 2-hydroxy acid dehydrogenases,[3] but this predicted enzymatic activity has not been demonstrated experimentally until recently.[2,4,5] Using pyruvate as a substrate, NADH as a cofactor and bacterially purified CtBP1 as the source of enzyme, studies from a number of labs have shown that CtBP1 can convert pyruvate to lactate, as measured by the conversion of NADH to NAD in a dehydrogenase reaction. Importantly, mutation of the conserved histidine

*Corresponding Author: Yang Shi—Department of Pathology, Harvard Medical School, Boston, Massachusetts, U.S.A., Email: yang_shi@hms.harvard.edu

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residue (amino acid 315), predicted to be critical for catalysis, completely abolished the dehydrogenase activity, strongly suggesting that this newly found enzymatic activity is intrinsic to CtBP1. X-ray crystallography provided further structural insight that substantiates the notion that CtBP1 is a dehydrogenase.[5,6] However, two important issues remain. First, the Km for CtBP-mediated dehydrogenation reaction is significantly higher than any bona fide dehydrogenases. It is therefore almost certain that pyruvate is not the physiological substrate of the CtBP proteins. It will be important to identify the physiological substrate, which constitutes an important piece of the missing puzzle. Second, the biological significance of this dehydrogenase activity is still unclear. Using mouse embryonic cells (MEF) carrying null alleles for both CtBP1 and CtBP2, Frisch, Goodman and colleagues found that the catalytically inactive CtBP mutant functions like the wildtype protein in its ability to restore repression of the CtBP target genes in vivo.[7] This finding suggested strongly that the dehydrogenase activity is not required, at least not directly, for CtBP-mediated transcriptional repression. A recent study of the drosophila CtBP suggests that NAD binding is important for CtBP-mediated repression after CtBP is recruited to the promoter.[8] Thus, it remains a challenge to decipher the role of the dehydrogenase activity of CtBP in vivo.

HPC2: A Chromodomain-Containing Sumo E3 Ligase that May Bind Methylated K27 of Histone H3

HPC2 is the human homolog of the Drosophila Polycomb (Pc) protein, which is a part of the Polycomb Group (PcG) protein complex. HPC2 has been found in the CtBP super complex[2] and has also been isolated as a CtBP-interacting protein in a separate two-hybrid screen.[9] HPC2 contains the CtBP-interacting PXDLS motif, suggesting that HPC2 directly interacts with CtBP. Recent studies showed that HPC2 is a Sumo E3 ligase for CtBP, and that CtBP sumoylation is important for CtBP nuclear localization.[10] It is unknown whether HPC2-mediated sumoylation also impacts other aspects of the CtBP complex, in addition to CtBP subcellular localization. HPC2 has also been implicated in tumorigenesis based on the observation that over-expression of HPC2 C-terminal deletion mutants induce oncogenic transformation and apoptosis.[11] It has been hypothesized that these mutants transform cells by interfering with the activity of the endogenous HPC2. It will be interesting to explore whether the transforming activity of the mutant HPC2 proteins is linked to the disruption of the Sumo E3 ligase activity of HPC2, which would be predicted to effect CtBP subcellular localization and probably CtBP complex formation as well. Lastly, HPC2 also contains a chromodomain located N-terminally. The prototypical chromodomain, as exemplified by HP1 protein, has been shown to bind methylated H3-K9. HP-1, together with the heterochromatin-specific H3-K9 methylase Suv-39, is believed to be responsible for heterochromatin propagation.[12-14] The Drosophila Pc protein binds tri-methylated histone H3 lysine K27.[15-17] Whether the chromodomain in human PC2 plays a similar role to coordinate CtBP-mediated transcriptional repression remains to be determined.

HDAC1/2 and G9a/HMTase1: Histone Deacetylases and Methylases

The class I histone deacetylase HDAC1 and 2 as well as the two highly related euchromatic histone methylases G9a and HMTase1[18] have also been identified as components of the CtBP1 complex. Interactions of CtBP with HDAC1 and 2 have also been reported by other studies.[19-21] While HDAC1/2 can mediate deacetylation reactions on a host of lysine (K) residues on histones, G9a/HMTase function as heterodimer, which mediates methylation of histone H3, predominantly on lysine 9 (H3-K9) and, to a lesser extent, on lysine 27 (H3-K27).[18,22] K9 is a critical amino acid residue on the tail of histone H3 where different modifications are correlated with different transcriptional activity. Specifically, H3-K9 acetylation is associated with active transcription while methylation at the same site is associated with heterochromatin or repressive euchromatin.[23] The fact that both histone deacetylases and methylases are present in the CtBP repressor complex suggests that CtBP may be able to convert an active chromatin

environment to a repressive one through a coordinated action of both HDAC1/2 and G9a/HMTase1. Indeed, in vitro results supported this argument demonstrating that CtBP complex can mount a two-step reaction (deacetylation followed by methylation) that converts histone H3 preacetylated at K9 to methylated H3.[2] Consistent with the in vitro result, TSA treatment inhibits H3-K9 methylation and shifts the balance of acetylation to methylation on H3-K9 at the E-cadherin target promoter. Similarly, RNAi knockdown of CtBP resulted in a decrease in the promoter occupancy of G9a/HMTase1, leading to a decrease in K9 methylation, but an increase in K9 acetylation and promoter activity. These findings strongly suggest that CtBP mediates transcriptional repression in part through the action of HDACs and HMTases.

LSD1: The First Histone Demethylase

LSD1 (Lysine Specific Demethylase 1) (alias KIAA0601, p110b, BHC 110 and NPAO) is a flavin-containing protein based on its ability to bind FAD.[24] LSD1 is a transcriptional corepressor and is a component of a number of corepressor complexes, including CtBP,[2] CoREST (KIAA0601)[25] and a subset of HDAC complexes (BHC110 or p110b).[24,26] Its sequence homology with amine oxidases predicts that LSD1 may catalyze oxidation reactions of biogenic amines including monoamine, polyamines or N-methylated protein substrates (such as histones).[27] A recent study provided convincing evidence demonstrating that LSD1 is a histone demethylase that displays substrate specificity towards mono or dimethylated K4 of histone H3.[28] Through a two-step electron transfer oxidation reaction, LSD1 removes mono- or di-methyl group from the mthylated histone H3K4 and leaves an intact unmethylated H3K4 behind. LSD1 cannot, however, catalyze demethylation of tri-methylated H3K4 due to the requirement of protonated nitrogen, which is lacking in the trimethylated substrates. Furthermore, the same study provided evidence supporting the model that LSD1 represses transcription by demethylating H3-K4, where methylation has been linked to active transcription.[29-33] Thus, transcriptional repression mediated by the CtBP super-complex appears to involve both the enzymes that confer modifications important for repression as well as an enzyme that removes modifications important for transcriptional activation.

CDYL: A Nuclear Enyol-CoA Hydratase Homolog

CDYL (ChromoDomain Y-Like) is another protein in the CtBP complex, which shares sequence homology with enyol-CoA hydratases/isomerases, which bind CoA and are enzymes involved in lipid metabolism.[34] CDYL is a ubiquitous protein but is related to the sperm-specific CDY, both of which may play an important role in spermatogenesis.[34] Consistent with the sequence homology prediction, CDYL has been shown to bind CoA but whether it has enyol-CoA hydratase/isomarase activity remains unknown.[35] When targeted to promoters, CDYL mediates transcriptional repression but it is unclear whether the enzymatic motif plays a role in this process.[35] Although CDYL does not have apparent sequence homology with histone acetylases (HAT), a recent study suggested that CDYL functions as a HAT with a preference for histone H4.[36] In another study, the authors claimed that they could not identify the H4-specific HAT activity for CDYL.[35] Further investigation is necessary to resolve this discrepancy. It also remains to be determined with respect to the role CDYL may play in CtBP-mediated transcriptional repression. For instance, does the chromodomain of CDYL1 plays a role in spreading the repressive chromatin through binding to methylated H3-K9 and the H3-K9 methylases G9a/EuHMTase1?

Other Components and Target Genes of the CtBP Super Complex

In addition to the six enzymes/potential enzymes discussed above, CoREST as well as a number of known or predicted DNA-binding proteins also copurified with CtBP.[2] CoREST is recruited by the zinc finger DNA-binding repressor REST for repression of neuron-specific gene transcription in nonneuronal cells.[37] The mechanism by which CoREST represses

transcription is unclear. In C. elegans, the worm CoREST homolog directly interacts with one of the worm LSD1 homologs SPR-5.[38] Both proteins appear to be involved in the Notch signaling pathway.[38,39] Interestingly, recent studies also indicate that CoREST may regulate the stability, as well as chromatin accessibility of LSD1.[39a,39b] These results suggest that CoREST plays an important bridging function between LSD1 and the chromatin.

Upon inspection, it becomes clear that the CtBP and the CoREST complexes share many of the same components. It is therefore possible that these two complexes are one of the same or close variants. Consistent with this idea, unpublished results (Shi lab) suggest that CtBP, in addition to repressing pro-apoptotic genes and several epithelial-specific genes,[7,40] also appears to be involved in repressing neuron-specific genes as do the other CtBP/CoREST complex components such as LSD1. This is expected if CtBP and the CoREST complexes are one of the same or are closely related. It should be noted that the situation in vivo is likely to be more complicated. Rosenfeld and Mandel labs showed by chromatin immunoprecipitation that not all CoREST components are located at all target promoters examined. Consistently, they found that some CoREST promoters are sensitive to the HDAC inhibitor TSA while others are not.[41] These findings suggest that in vivo regulation of promoter occupancy of these complex components is likely to be highly dynamic.

Summary and Perspectives

The recent biochemical /proteomic approach has identified a CtBP super complex that has many exciting features. This complex can carry out a number of enzymatic reactions, all of which contribute to the establishment of a repressive chromatin environment. Specifically, the HDACs and the H3K9-specific histone methylases can convert acetylated K9 to methylated K9, thus providing the modification that is crucial for transcriptional repression. LSD1, which is the first histone demethylase ever identified, functions to demethylate H3K4, where methylation is associated with active transcription. These studies suggest that establishing a repressive chromatin environment is likely to involve multiple enzymes mediating reactions conferring repressive modifications as well as removing modifications important for active transcription. What remains to be accounted for are the dehydrogenase activity of CtBP and the role of CDYL, i.e., whether CDYL is an enzyme and if so whether the enzymatic activity plays a role in transcriptional repression. It is an interesting puzzle that CtBP is a dehydrogenase but this enzymatic activity appears to be dispensable for transcriptional repression.[7] LSD1 catalyzes demethylation via an oxidative reaction, which results in formaldehyde and H_2O_2.[28] It is tempting to speculate that the dehydrogenase activity of CtBP may play a role in the metabolic conversion of formaldehyde or H_2O_2. It is important to determine whether principles in metabolic pathways are also applicable in the nucleus, where many seemingly independent reactions are in fact coupled to provide efficient epigenetic gene regulation.

A complex transcription machine such as the CtBP super-complex is bound to be subjected to regulation under different physiological and pathological conditions. As discussed, a regulatory mechanism that is built into the CtBP complex is sumoylation mediated by HPC2.[10] In contrast to HPC2, which helps retain CtBP in the nucleus, neuronal nitric-oxide synthase binding to CtBP promotes CtBP relocalization to the cytosol.[42,43] CtBP activity is also regulated by phosphorylation[44,45] as well as by metabolic state of the cell due to fluctuation of the NADH level in the nucleus.[46] NAD and FAD are the two main electron carriers in the cell. Importantly, two components of the CtBP complex, i.e., CtBP and LSD1, are subjected to NAD and FAD regulation. Thus, the CtBP complex may represent an important direct link between metabolism and transcription. Finally, other components of the CtBP complex are likely to be regulated by various post-translational modifications and protein-protein interactions as well. For instance, LSD1 chroamtin accessibility and activity are regulated by its interacting protein CoREST, which is another component of the CtBP supercomplex (refs. 1,2), and BHC80 (a PHD domain-containing protein), respectively (ref. 1). regulated by its interacting protein CoREST, which is another component of the CtBP super complex (Shi lab, unpublished results).

The identification and characterization of the CtBP super-complex has already provided exciting and important insights into eukaryotic transcriptional regulation. Future experiments promise to continue to shed light on fundamental transcriptional mechanisms and how metabolic and oncogenic signals may impact these basic regulatory functions in development and diseases.

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CHAPTER 9

Structural Determinants of CtBP Function

James R. Lundblad*

Abstract

The structural characteristics of the CtBP family of transcriptional corepressors suggest an additional role for coenzyme nicotinamide adenine dinucleotide in the repression of gene expression. Remarkably, CtBP orthologues are unique among transcriptional regulators in that they display striking primary sequence and structural similarity to the D-isomer specific 2-hydroxyacid dehydrogenase class of enzymes. Recent structural studies of rat CtBP/BARS and human CtBP1 provide insight into the role of pyridine dinucleotide binding in regulation of CtBP quaternary structure, and corepression activity through association with –PXDLS-containing targets.

Introduction

In addition to a central essential role in metabolism as a carrier of reducing equivalents, the nicotinamide adenine dinucleotide coenzymes (NAD and NADP)[a] play important roles in cellular signaling, also serving as substrates for covalent protein modifications as well as precursors to the synthesis of intracellular calcium mobilizing second messenger molecules (reviewed in ref. 1). Moreover, recent studies implicate NAD in a variety of nuclear transactions. For some of these processes, a role for NAD as a recipient or donor of reducing equivalents seems to be important. For example the DNA binding of Clock-BMAL1 and NPAS2-BMAL1 heterodimers is regulated by the ratio of reduced to oxidized NAD(P).[2,3] In contrast, other NAD-dependent nuclear processes result in a net consumption of NAD, cleaving the N-glycosidic bond between nicotinamide and ADP-ribose. DNA damage response pathways activate poly(ADP-ribose) polymerase-1 (PARP-1) leading to the addition of polymers of ADP-ribose to a number of nuclear proteins.[4] In another example, the chromatin-associated silencing protein Sir2 (Silencing information regulator 2) functions as a NAD-dependent protein deacetylase acting on histones as well as a variety of transcription factors, generating O-acetyl-ADP-ribose and nicotinamide as products.[5]

The structural characteristics of the CtBP family of transcriptional corepressors suggest an additional role for the coenzyme NAD in transcriptional regulation. The first CtBP homologue (CtBP1) was identified as a binding partner for the adenoviral transforming protein E1A[6] however CtBP homologues have been implicated as cofactors for a number of cellular

[a] By convention, NAD(P) refers to nicotinamide adenine dinucleotide (phosphate) without reference to the dinucleotide oxidation-reduction state. $NAD(P)^+$ specifies the oxidized form, whereas NAD(P)H specifies the reduced form.

*Corresponding Author: James R. Lundblad—Division of Molecular Medicine, Department of Medicine, and Biochemistry and Molecular Biology, Oregon Health & Sciences University, HRC-3, 3181 S.W. Sam Jackson Park Road, Portland, Oregon 97239, U.S.A.
Email: lundblad@ohsu.edu

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transcriptional repressors.[7,8] CtBP target proteins share a common consensus motif, related to -PXDLS-, which is essential for the recruitment of CtBP. Deletion of this motif in these proteins abrogates CtBP binding and, at least partially, their transcriptional repressor activities. Remarkably, CtBP orthologues lack prototypical features associated with typical transcriptional regulatory proteins, but are unique among transcriptional regulators in that they display striking primary sequence and structural similarity to the D-isomer specific 2-hydroxyacid dehydrogenase class of enzymes. These proteins are also unusual in that CtBP homologues appear to harbor dual cytoplasmic and nuclear functions. In addition to a proposed widespread roles in transcription, nonnuclear functions of CtBP homologues are suggested by the recognition that the Brefeldin A ribosylation substrate or BARS-50, a component of the Golgi tubule fission complex, is virtually identical to CtBP1.[9] The identification of RIBEYE, a CtBP2 splice variant as a component of the ribbon synapse[10] additionally supports the concept of functional complexity beyond the nucleus for this family of proteins.

What is the significance of the dehydrogenase homology in the transcriptional repression functions of CtBP? Current studies suggest two general models. In the first, the dehydrogenase domain serves a structural or scaffolding function independent of an enzymatic activity for the recruitment of other coregulatory proteins, including proteins with chromatin remodeling activities. In support of this mechanism, numerous laboratories have found an association of CtBP1 and CtBP2 with histone deacetylases (reviewed in ref. 8), and a macromolecular corepressor complex comprised of CtBP in association with histone deacetylase, histone methyltransferase and demethylase activities as well as other corepressors has been purified from HeLa cells.[11] Notably, dinucleotide promotes the interaction of CtBP with E1A and other targets[12-14] and facilitates the dimerization of CtBP.[15,16]

An alternative but not necessarily exclusive model is that CtBP homologues harbor an intrinsic enzymatic activity essential for these diverse functions. Although several laboratories have demonstrated weak dehydrogenase activity with a surrogate substrate, the identities of bona fide substrate(s) for any oxidation-reduction enzymatic activity of CtBP remain elusive. Consequently, how CtBP might participate as a dehydrogenase in the process of transcriptional repression remains speculative. Moreover the link between a putative dehydrogenase activity and one activity proposed for CtBP/BARS, an acyltransferase activity employing acyl-CoA in the conversion of lysophosphatidic acid into phosphatidic acid[17] is unclear. In this chapter, we explore the structural features of CtBP and the relationship to the known biochemical properties of this unusual corepressor protein family.

CtBP: General Structural Considerations

Schaeper et al[6] first noted the similarity of CtBP to the D-isomer specific 2-hydroxyacid dehydrogenases with the cloning of CtBP1. This family of proteins, with members conserved from prokaryotes to higher metazoan organisms, includes formate dehydrogenase (FDH), D-glycerate dehydrogenase (D-GDH), D-3-phosphoglycerate dehydrogenase (3PGDH), D-lactate dehydrogenase (D-LDH), and D-2-hydroxyisocaproate dehydrogenase (D-HicDH).[18] These enzymes are typically composed of 2 to 4 identical polypeptide subunits, each subunit composed of 2 or more domains.

To date, three crystallographically determined structures of CtBP homologues have been reported, including a the structure of a core domain of human CtBP1 refined to 1.95 angstrom (Å) resolution (Protein Data Bank entry 1MX3),[13] and the closely related rat CtBP/BARS protein in complex with NAD at 2.3 Å resolution (Protein Data Bank entry 1HKU).[14] The latter report also includes the solution of a ternary complex of CtBP/BARS with NAD and a model PXDLS peptide (PIDLSKK) at 3.1 to 3.5 Å resolution (Protein Data Bank entry 1HL3) (Fig. 1). Human CtBP1 and rat CtBP/BARS have a high degree of homology (97% identity), differing in sequence primarily due to an amino terminal extension of CtBP1 not present in CtBP/BARS.[9,14] Each of these structures share overall structural homology with the core domains of the D-2-hydroxyacid dehydrogenases.

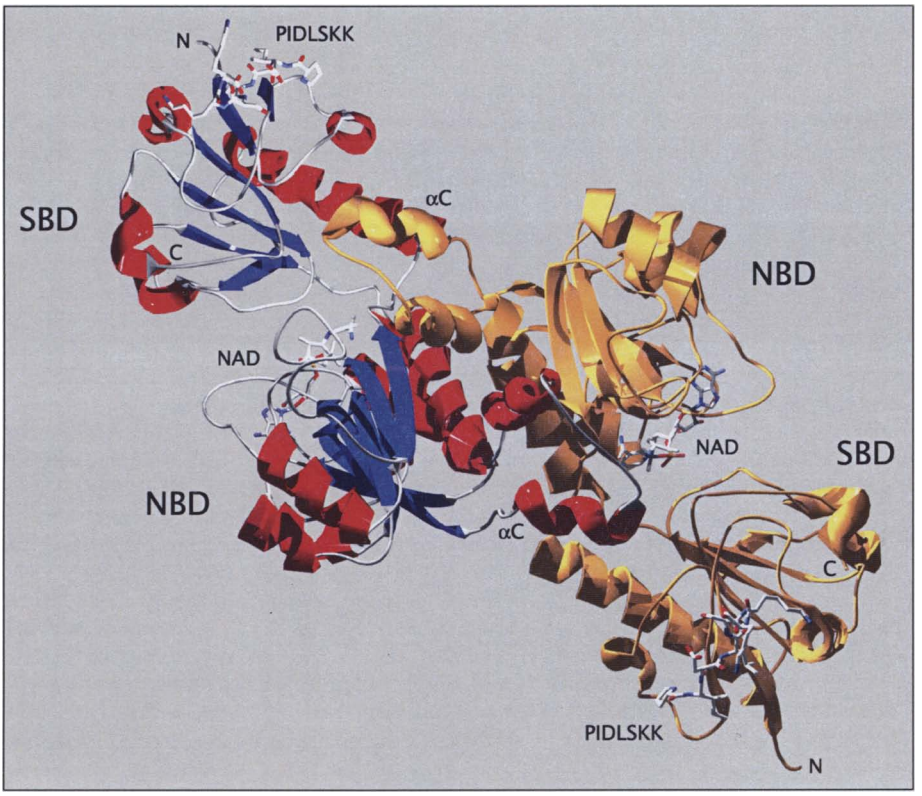

Figure 1. Structure of rat CtBP/BARS ternary complex. A ribbon diagram of rat CtBP/BARS (residues 15-345) bound to NAD, in complex with a model heptamer peptide corresponding to a consensus binding sequence (-PIDLSKK-) showing a 2-fold crystallographic axis between the two monomers (Protein Data Bank entry 1HL3).[14] Blue ribbons represent β-strand, and red ribbons α-helix secondary structure. One of the dimer pair is represented in gold for clarity. The structures of NAD and PIDLSKK peptide bound to each monomer are indicated in stick representation. The substrate-binding domain (SBD), the nucleotide-binding domain (NBD), and amino- (N) and carboxyl-terminal (C) residues are indicated.

Each structure was determined from a C-terminally truncated form, encompassing all but the last 80-90 amino acids but corresponding to the structurally homologous substrate-binding domain (SBD), and nucleotide-binding domain (NBD) characteristic of the D-2-hydroxyacid dehydrogenases (Fig. 1). Like other dehydrogenases, these structures demonstrate that CtBP homodimerizes through the dinucleotide-binding domain, forming an extensive, largely hydrophobic dimerization interface, which buries ~3200 Å^2. Previous interaction mapping studies suggested dimerization occurs through this domain.[19] In these structures, a deep cleft separates the SBD from the NBD, corresponding to a putative active site, and the dinucleotide-binding pocket. The C-terminal domain of CtBP, missing from all of the proteins used in these experiments, may correspond to the C-terminal regulatory binding domain (RBD) of 3PGDH[20] although little is known about the function of this portion of CtBP. Nevertheless, the PDZ domain of neuronal nitric oxide synthase binds near the C-terminus[21,22] and this is also the site of SUMOylation,[22] an apparent determinant of nuclear localization.

The CtBP dimer forms an elongated structure with the substrate-binding domains of each respective monomer, and thus each PXDLS docking site, at opposite extremes (Fig. 1). As has been observed for other dehydrogenases, the topology of the CtBP substrate-binding domain

is composed of a discontinuous peptide sequence composed of residues from both the amino (CtBP1 residues 27-121; BARS residues 1-112) and the carboxyl terminus (CtBP1 residues 327-352; BARS residues 309-350) of the protein. As will be discussed in the next section, this domain harbors determinants for recruitment of PXDLS-containing target proteins, thus the homodimeric complex of CtBP potentially contacts two PXDLS-containing targets at opposite poles. The discontinuous topology of this domain complicates the interpretation of previous deletional mutagenesis of the amino terminus.[23-25] The functions of the globular substrate-binding domain may not be strictly separable from those of other domains of CtBP by simple deletion due to this structural organization.

Is CtBP a functional dehydrogenase? For the D-2-hydroxyacid dehydrogenases, catalytic activity apparently proceeds through a "proton shuttle" between a histidine and a carboxylic acid residue (i.e., glutamate or aspartate) with the transfer of hydride ion between the substrate and coenzyme. An arginine residue located within proximity to the active site in 3PGDH interacts with the substrate carboxylic acid during catalysis.[26] These residues are conserved in all D-2-hydroxyacid dehydrogenases.[27] All mammalian CtBP orthologues as well the *Drosophia* CtBP homologue also include these residues (hCtBP1 residues H315, E295, R266; corresponding residues in CtBP/BARS include H304, E284, and R255; (Fig. 2) indicating CtBP might retain oxo-reductase enzymatic activity. The role of this proposed dehydrogenase activity in repression remains controversial. In some contexts, CtBP-dependent transcriptional repression depends on these putative catalytic residues[13] whereas these residues are dispensable in other experimental paradigms.[28-31]

In spite of the conservation of active site residues in CtBP, bona fide substrate(s) for the dehydrogenase activity of CtBP remain unidentified. Based on modeling, Kumar et al[13] suggest that CtBP binds a 2-hydroxy acid smaller in size than isocaproate. Several laboratories have measured a weak dehydrogenase activity of CtBP1 directed against pyruvate, with the concomitant oxidation of NADH to NAD^+ as measured by a loss of absorbance at 340 nm.[13,16,32] The lability of NADH under aqueous conditions at neutral pH, and the extraordinarily high concentrations of CtBP required for the detection of this activity makes interpretation and the physiological significance of these experiments uncertain.

Dinucleotide Binding by CtBP

The dinucleotide-binding domain with an evolutionarily conserved structure forms the core homology domain among these proteins. The NAD(P)-binding fold consists of two units of a mononucleotide-binding motif termed the Rossmann fold, a conserved structural domain composed of three parallel β strands interconnected by α helices, forming a parallel twisted β sheet flanked by α helices with a βαβαβ topology. In the dehydrogenase domain, each repeated βαβαβ structural element binds a mononucleotide component of the NAD(P) coenzyme.[33] These proteins characteristically harbor a glycine-rich loop that connects the C-terminus of the first β sheet of the fold (by convention, β1) with the N-terminus of the first α helix (termed αA) (reviewed in see refs. 34,35) in the first βαβ unit. This conserved "fingerprint" sequence, typically a variant of G/AxGxxG(17x)D (where x is any amino acid), serves as a phosphate-binding motif, interacting with the connecting pyrophosphate moiety of NAD (Fig. 2). Each of the CtBP structures comprises a well-conserved dinucleotide-binding signature motif (located between G181 and D204 of hCtBP1; and G170 and D193 of CtBP/BARS), corresponding to βA and αD portion of these structures.[13,14] The structure of CtBP demonstrates the rationale for the apparent specificity of binding for NAD over NADP.[12] Aspartate 204 in CtBP1 (D193 in CtBP/BARS) forms a hydrogen bond to the O2' and O3' atoms of the adenine-ribose moiety and would prevent binding of NADP(H) by electrostatic repulsion of the 2'-phosphate (Fig. 2). Dehydrogenase domains that bind NADP lack negatively charged side chains at the analogous position to accommodate the phosphate.[33,34]

What are the consequences of NAD binding on the tertiary structure of CtBP? Incubation of CtBP with NAD decreases sensitivity to limited proteolysis[12,13] suggesting an alteration in

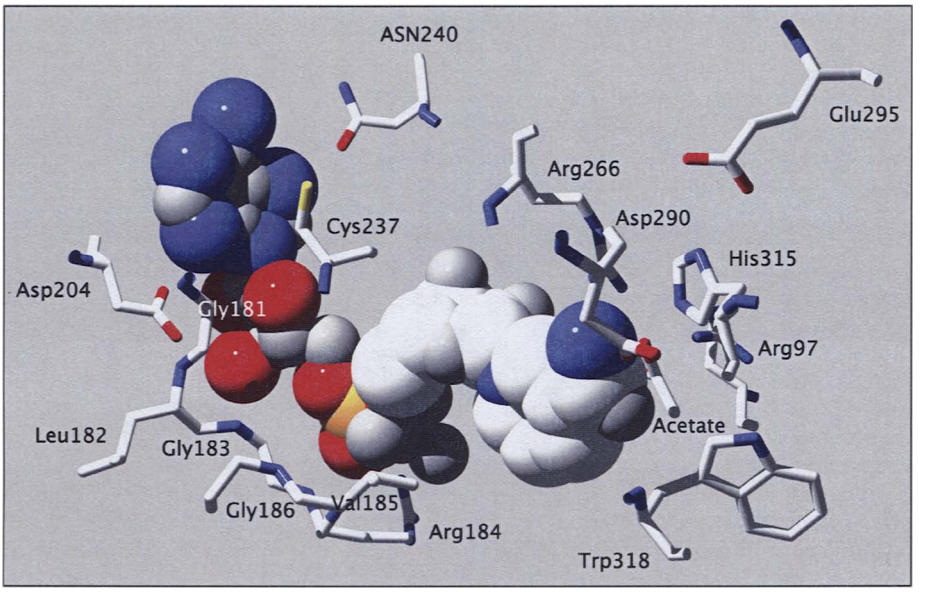

Figure 2. NAD binding pocket of human CtBP1. Side chain and peptide backbone of critical residues the NAD binding pocket of hCtBP1 are shown in stick representation (backbone oxygens are omitted for clarity) (Protein Data Bank entry 1MX3).[13] NAD is shown as a space-filling model. Glu296, His315, and Arg266 are presumed catalytic residues conserved among D-2-hydroxyacid dehydrogenases. A conserved glycine-rich loop between Gly181 and Gly186 of hCtBP1 interacts with the pyrophosphate moiety of NAD. Substitution mutations within this region diminish or abolish NAD binding and enzymatic activity of related dehydrogenases. In addition to interactions with backbone amides of Arg184, Val185, and Trp318 and carbonyl groups of Cys237 and Thr264 (not shown), side chains of Arg184, Asp204, Asn240 and Asp290 additionally stabilize NAD binding,. An acetate incorporated from crystallization solutions hydrogen bonded to His315, Arg266 and Arg 97 is shown in the active site adjacent to the nicotinamide ring.

protein conformation. The CtBP structures determined to date all include bound NAD. In fact Nardini et al[14] did not include NAD^+ in crystallization solutions yet found it in the final structure suggesting NAD copurified with the bacterially expressed CtBP/BARS used for crystallography experiments. Comparison of the NAD-bound CtBP structures with *apo* forms of other dehydrogenases suggest that the NAD bound form adopts a "closed" conformation, facilitated by intersubunit interactions at the dimerization interface. Although the corresponding portion of CtBP1 was not included in the protein used for structure determination, CtBP/BARS residues 140-154 (αC) of the one subunit come in close contact with the β1-α1 loop (residues 21-27) of the substrate-binding domain of the opposite subunit (Fig. 1). Intersubunit interactions or domain swapping induced by NAD binding may in part account for NAD-dependent dimerization of CtBP1[16] and CtBP2.[15] Induction of dimerization in fact may account for NAD-induced changes in protease sensitivity.

Is CtBP a metabolic sensor, discriminating the oxidation state of NAD? Most studies to date employ NAD- or NADH-stimulated association with a PXDLS-containing target as a proxy for the measurement of dinucleotide binding. An initial report demonstrated large differences in the efficacy of NADH vs. NAD^+ in promoting the interaction with E1A,[12] while others found little or no difference.[13,16] Studies directly comparing binding affinities of NADH and NAD^+ are notably absent. Fjeld et al[36] utilized a method based on fluorescence resonance energy transfer (FRET) between intrinsic tryptophan residues of CtBP and bound NADH to calculate an apparent K_d of 66 nM for NADH, a value in agreement with the approximate

concentration efficacious for enhancing binding of CtBP to E1A.[12] The donor fluorophore in these experiments was attributed to tryptophan 318 predicted to lie in close proximity to the dinucleotide binding pocket[13] since mutation of this tryptophan to phenylalanine (W318F) eliminates the FRET signal seen upon addition of NADH. Nevertheless, analysis of the crystal structure of CtBP1[13] suggests that Trp318 also may stabilize the interaction with dinucleotide by peptide backbone contributions to dinucleotide binding. Hence, the loss of FRET signal in these experiments might reflect disruption of NADH binding by this mutation. Competition experiments, fluorescently determining displacement of bound NADH with NAD^+, estimate the K_d for oxidized NAD^+ in the range of 8-16 μM.[36] In contrast, direct binding experiments demonstrate that the CtBP2 portion of RIBEYE (the B domain) binds oxidized NAD with approximately 10-fold higher affinity (estimated K_d of ~1.3 μM).[10]

Structural studies do not resolve this discrepancy however, since these crystallography experiments cannot discern the redox state of bound NAD. As discussed above, NAD co-crystallized with CtBP/BARS apparently entered the structure from the expression and purification from of the recombinant protein from *E. coli*. Based on differences in orientation of the nicotinamide carboxamide in this structure relative to that of the CtBP1 structure, these authors[14] suggest that the copurified NAD corresponds to the reduced form, arguing that positively charged residues in the active site (R255/266 and H304/315) might favor binding of the reduced form over oxidized, charged NAD^+. As precedence for this idea, copurification of tightly associated NADH has been reported for 3PGDH.[37] Furthermore, a fraction of partially purified bacterially expressed CtBP1 may also contain contaminating intrinsically-bound NADH.[36] Differences in the source of recombinant protein may also contribute to these discrepancies since some experiments describe proteins derived from bacterial or insect cell expression systems, whereas others employ in vitro transcribed/translated protein products in binding experiments. Direct comparative binding studies using highly purified recombinant protein will unambiguously resolve the question of differential binding of NAD^+ and NADH.

Interaction of CtBP with Binding Partners

What are the consequences of NAD binding for the recruitment of CtBP to binding partners? In the current model NAD occupancy induces a conformation that is permissive for the association of CtBP with binding partners, first suggested by the observation that inclusion of NAD stimulated the binding of CtBP to E1A.[12] A number of studies have subsequently demonstrated that dinucleotide-stimulates PXDLS-target binding (for example see refs. 13,15,32). In spite of this, numerous previous studies indicate that CtBP binds with high affinity to E1A in the absence of dinucleotide, and studies of dinucleotide binding mutants indicate that NAD binding and target acquisition are separable (for example see ref. 14).

The prevailing model predicts that NAD binding mutants will act as repressors as GAL4 DNA binding protein fusions, since fusion with a heterologous DNA binding domain would bypass NAD-dependent recruitment.[12,31] In support of this model, transient expression experiments indicate that selective point mutations of the NAD binding fold do not alter repressor activity of GAL4-CtBP.[31] In contrast, other more disruptive mutations clearly abrogate CtBP repressor activity[13] which might suggest an alteration in an intrinsic repression function apart from NAD-dependent recruitment. Interestingly, a dCtBP NAD-binding mutant as a GAL4-fusion fails to repress a reporter construct in the *Drosophila* embryo, suggesting this activity may require packaging of the reporter in the context of chromatin.[30]

Is NAD binding structurally linked to PXDLS recruitment? The structure of the ternary complex of CtBP/BARS:NAD with a model peptide comprised of a consensus PXDLS motif indicates that these targets interact with the amino-terminal substrate-binding domain.[14] The model peptide, -PIDLSKK-, binds by docking of the isoleucine and leucine side chains into a hydrophobic cleft formed by a parallel β-sheet composed of the β1 and β2 strands with the α2 helix of the amino terminus of the substrate-binding domain. Comparison between the structures

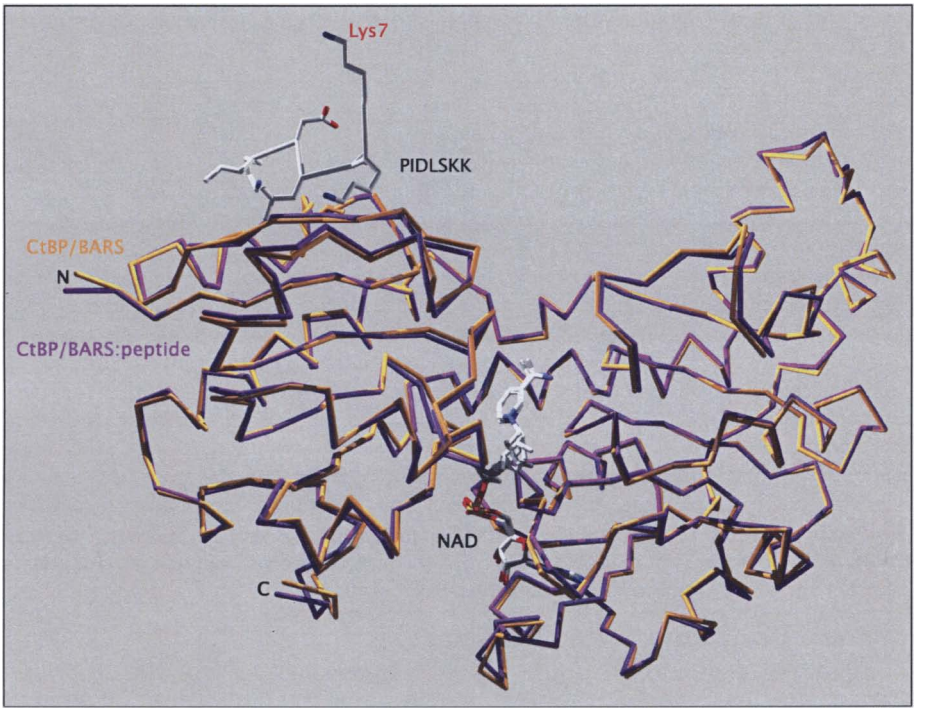

Figure 3. Overlay of CtBP/BARS binary complex and ternary complex. Cα trace of the monomeric binary complex of CtBP/BARS with NAD is shown in gold (Protein Data Bank entry 1HKU), with that of the ternary complex (CtBP/BARS:NAD with bound PIDLSKK peptide) in purple (Protein Data Bank entry 1HL3).[14] Amino- (N) and carboxyl-terminal (C) residues are indicated.

of the binary and ternary complexes demonstrates that binding of the peptide is not associated with significant structural alterations in CtBP, and that the PXDLS binding site does not directly contact the NAD binding site (Fig. 3), in contrast to an analysis of E1A binding activities of core dehydrogenase mutants previously reported.[13]

A recent report suggests that CtBP may employ a different mode of NAD(H)-dependent regulation in an interaction with the bromodomain of the transcriptional coactivator p300.[38] Bromodomains serve as docking modules for acetylated lysine residues, promoting the association of bromodomain-containing proteins with modified histones and other acetylated proteins.[39] The sequences of bromodomains from a number of transcriptional regulators, including CBP, p300, and GCN5, contain a consensus –PMDLS- motif, however an analysis of the yeast GCN5 structure[40] indicates this motif may have limited surface accessibility (Protein Data Bank accession 1E6I). Nevertheless, these authors demonstrate that CtBP binds the p300 bromodomain in a manner that depends on the –PMDLS- sequence, and inhibits the transcriptional activity of p300. Furthermore they demonstrate that the interaction of CtBP with the bromodomain dissociates acetylated histones, apparently through an interaction inhibited by NADH.[38] The Hdm2 interaction with CtBP also shows this unusual mode of regulation by NADH.[41] These results suggest that a subset of partners may exploit a surface of CtBP for interaction different from that utilized for the prototypical PXDLS-containing targets.

Posttranslational modification by lysine acetylation within the CtBP interaction motif may also regulate the interaction of CtBP with targets. Although mutations within the core pentapeptide –PXDLS- motif profoundly disturb the interaction with CtBP,[6,42,43] a systematic

evaluation of the association of CtBP with peptides derived from a number of cellular and viral targets indicate that sequences outside the core -PXDLS- motif influence binding affinity.[44] Acetylation of 12S E1A at a lysine residue adjacent to the core pentapeptide (-PLDLSCK^{239}) by the acetylases p300, CBP, and P/CAF attenuates its ability to repress CBP/p300-dependent transcriptional coactivation.[45,46] For related sequences in many cellular CtBP targets, a lysine residue at this position is also present (reviewed in ref. 8). The role of this lysine in regulating the interaction of these targets with CtBP is controversial however. In 12S E1A, this lysine functions as essential residue in the carboxyl-terminal nuclear localization signal, and lysine acetylation distributes a fraction of E1A to the cytoplasm.[46] Consequently, acetylation at this lysine quantitatively disrupts interaction of E1A with the nuclear import receptor Importin-α but does not impair CtBP binding, in contrast to the conclusions of a previous study.[45]

Analysis of the CtBP/BARS:NAD:peptide structure provides some insight into the role of this residue in CtBP binding. The model peptide resembles the E1A site with a lysine at position 7 (-PIDLSKK-), yet this residue does not make contact with the docking site in the largely hydrophobic binding cleft of the substrate-binding domain, nor does it contact other determinants in this structure[14] (Fig. 3). These results are consistent with the systematic evaluation of the CtBP binding properties of the carboxyl-terminus of E1A which demonstrated the insensitivity of this lysine to substitution,[43] and acetylation.[46] In the context of a centrally located PLDLS motif, flanking sequences may participate in other contacts with CtBP and thus acetylation may have more profound effects on CtBP recruitment, as has been reported for acetylation of the nuclear hormone receptor corepressor RIP140.[47]

Summary and Unresolved Questions

The structures reported for CtBP1 and BARS provide insight into the determinants of pyridine dinucleotide binding and target recognition, and hint at the potential roles of the conserved dehydrogenase homology domain in the processes of transcriptional repression. In spite of the details revealed by these structural models, much remains unresolved.

What structural alterations does CtBP undergo on NAD binding? A structure of the *apo* form of CtBP, in the absence of bound NAD, has not been determined to date. The solution of the structure of CtBP in the absence of NAD will help resolve the question of linkage between binding of NAD and the association of PXDLS-containing targets. Furthermore, detailed thermodynamic evaluation and direct comparison of the binding of oxidized and reduced NAD remains necessary to resolve the controversial model of CtBP as a redox-sensitive transcriptional corepressor. Molecular modeling may facilitate identification of bona fide substrates and other ligands for CtBP, including characterizing determinants for acyl-CoA binding.[14] Ultimately, this could lead to the generation of designer pharmacological modulators of CtBP activities for in vivo use.

How are CtBP1 and CtBP2 different from a structural standpoint? The core domains of each protein are highly homologous, yet the amino- and carboxyl-terminal domains diverge. Structures including the carboxyl-terminal domains of these proteins may provide insight into the roles of these domains in regulation of CtBP function. Finally, the characterization of CtBP in a complex with a protein rather than peptide binding partner will clarify the role of determinants outside of the core pentapeptide interaction motif in recruitment of a CtBP corepressor complex, and its regulation by pyridine dinucleotides.

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CHAPTER 10

CtBP3/BARS and Membrane Fission

Stefania Spanò, Cristina Hidalgo Carcedo and Daniela Corda*

Abstract

CtBP3/BARS was the third protein of the CtBP (C-terminal binding protein) family to be identified. It was initially isolated as a 50-kDa cytosolic protein during the characterisation of the molecular targets of the toxin brefeldin A (BFA). As this protein is a substrate of BFA-dependent ADP-ribosylation, it was initially named BARS-50 (BFA-dependent ADP-ribosylation substrate), or BARS. After its purification and cloning, the protein was shown to be the third member (hence CtBP3/BARS) of the CtBP transcription corepressor family of proteins, sharing a high degree of aminoacid identity with CtBP1 (97%). CtBP3/BARS induces membrane fission in isolated Golgi membranes and is necessary for the fragmentation of the Golgi complex that occurs at the beginning of mitosis; its direct role in transcription regulation has not yet been specifically investigated. The CtBPs are thus a multifunctional protein family that can modulate both nuclear and cytosolic functions.

CtBP3/BARS As a Substrate of BFA-Dependent ADP-Ribosylation

As indicated above, CtBP3/BARS was initially identified as the 50-kDa substrate of BFA-dependent ADP-ribosylation.[1,2] BFA is a fungal toxin[3] that induces a very rapid block of secretion.[4,5] As with other toxins, BFA has been widely used over the last twenty years as a tool to elucidate the molecular mechanisms of transport. In addition to blocking secretory traffic, BFA induces a dramatic morphological reorganisation of the Golgi complex and the redistribution of both resident and cargo proteins from the Golgi complex to the endoplasmic reticulum (ER).[5-8] Moreover, BFA affects the morphology and function of the endosomal/lysosomal compartments by inducing the tubulation and fusion of the endosomal membranes.[9,10]

The first molecular target of BFA was identified as the exchange factor for the small GTPase ARF.[11,12] In 1994, we showed that BFA was also able to induce the ADP-ribosylation of two cytosolic substrates of 38 kDa and 50 kDa.[2] The 38-kDa substrate was identified as an isoform of glyceraldehyde 3-phosphate dehydrogenase (GAPDH),[2] a glycolytic enzyme with multiple cellular functions;[13] however, only a small percentage of the total cellular GAPDH is modified by BFA.[2] The 50-kDa substrate (CtBP3/BARS) was shown to contribute to the ability of BFA to disassemble the Golgi complex, indicating a possible role for this protein in the control of the structure of this organelle.[14] In order to obtain the protein sequence, CtBP3/BARS was purified from rat brain cytosol by following its ADP-ribosylation in the presence of [^{32}P]-NAD through four chromatographic steps.[15] After an 800-fold enrichment of cytosolic CtBP3/BARS, it was separated by two-dimensional gel electrophoresis, trypsin-digested, and subjected to protein microsequencing. The peptide sequences obtained from microsequencing were used to generate two probes to screen a rat brain cDNA library. One clone (GenBank Accession Number AF067795) contained a full-length open reading frame (ORF) that coded for a

*Corresponding Author: Daniela Corda—Department of Cell Biology and Oncology, Consorzio Mario Negri Sud, 66030 Santa Maria Imbaro (Chieti), Italy. Email: corda@negrisud.it

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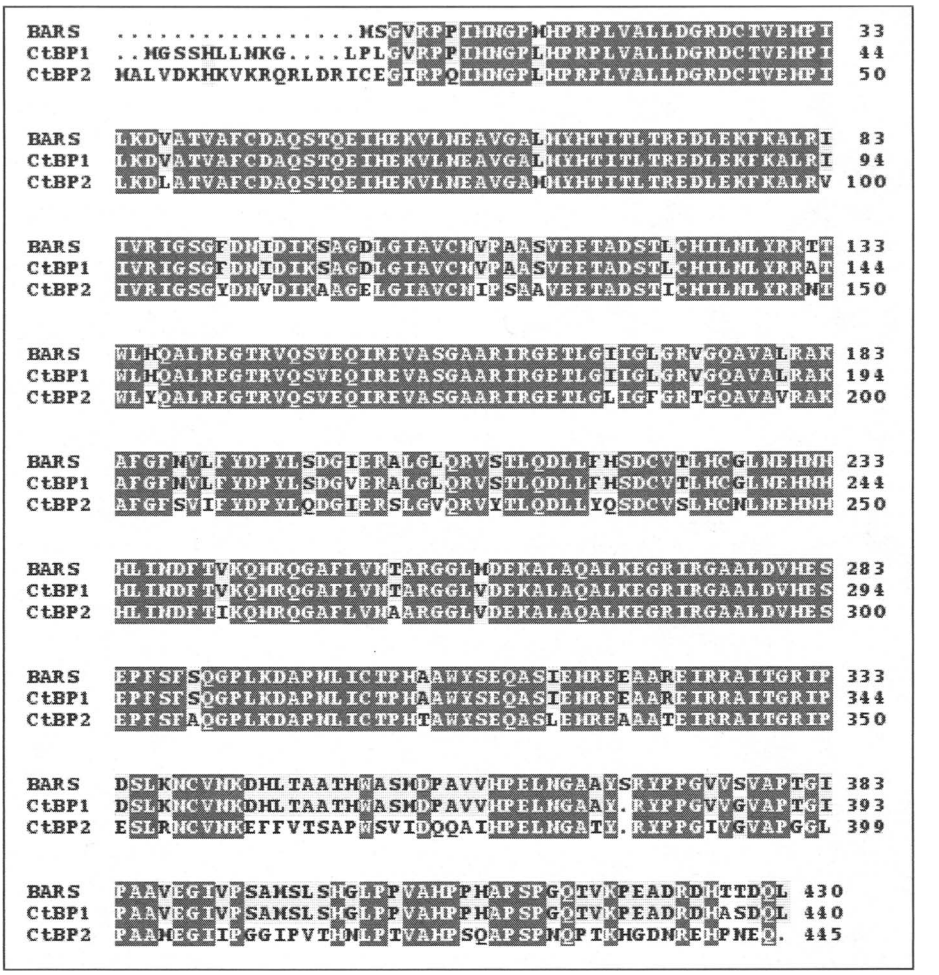

Figure 1. CtBP3/BARS belongs to the CtBP family. CtBP3/BARS is aligned with mouse CtBP1 (accession number AJ010483) and mouse CtBP2 (accession number AF059735). Identical residues are in white on a dark grey background; conserved residues in the sequences are in black on a light grey background. CtBP1 and CtBP3/BARS are also now referred to as CtBP1-L and CtBP1-S, respectively, as they represent the long and short splice variants deriving from the CtBP1 gene.

430-aminoacid protein with a predicted mass of 47 kDa. When transfected into COS7 cells, the cloned cDNA expressed a 50-kDa cytosolic protein that proved to be a substrate of ADP-ribosylation induced by BFA. The use of antibodies raised against CtBP3/BARS peptides or against a GST-CtBP3/BARS fusion protein in immunoprecipitation experiments also confirmed that the cDNA isolated in the screening actually coded for CtBP3/BARS.[15]

Rat CtBP3/BARS is highly similar to CtBP1 and CtBP2. These latter two proteins have been cloned in human and mouse. At the aminoacid level, CtBP3/BARS shares a 97% identity with human and mouse CtBP1 (accession numbers: U37408 and AJ010483), and a 79% identity with human and mouse CtBP2 (accession numbers: AF016507 and AF059735) (Fig. 1). A significant difference between CtBP1 and CtBP3/BARS resides the N-terminal portion, where the two proteins differ in sequence and length. Another sequence feature particular to

CtBP3/BARS is the presence of a serine in position 369 that has no correspondence with the other two CtBPs. This insertion may represent a significant difference in terms of the secondary and tertiary structures of these three proteins.[15]

At the nucleotide level, the rat CtBP3/BARS cDNA is 94% identical to mouse CtBP1 (86% to human CtBP1) and 72% identical to human and mouse CtBP2. Interestingly, a 192-nucleotide sequence in the CtBP3/BARS cDNA, including its translation initiation codon, is absent in the CtBP1 and CtBP2 cDNAs. Comparison of the cDNA sequences with the available genomic sequences indicated that both CtBP1 and CtBP3/BARS are expressed from the same gene located on chromosome 4p16 and that the 192-nucleotide sequence represents an additional exon included in CtBP3/BARS mRNA, but not in CtBP1 mRNA (our unpublished results).[16] CtBP1 and CtBP3/BARS are thus alternative splice variants. The CtBP3/BARS specific exon is also present in several mouse and human sequences of the EST database, indicating that CtBP3/BARS is not an isoform exclusive to rat, but is likely to be expressed in all mammals. Considering that CtBP3/BARS was the third of the CtBP isoform identified, we now refer to it as CtBP3/BARS in order to adhere to the family name and at the same time to keep the descriptive name for its features. The EST database was also searched for rat sequences that could code for CtBP1, but no such sequences were found (our unpublished results).[16] However, given the limited number of rat EST sequences present in the database, this finding does not exclude the possibility that CtBP1 is also expressed in rat.

Interestingly, CtBP1 and CtBP2 are also substrates for the ADP-ribosylation induced by BFA,[16] suggesting that this ADP-ribosylation can also regulate the corepression activity of CtBP1 and CtBP2, in a similar way to its inhibition of CtBP3/BARS function.[15,17]

The CtBP3/BARS Fissioning Activity

Membrane Fission

Membrane fission is an essential event in intracellular membrane traffic. In order to transport proteins and lipids from donor to acceptor membranes, membranous carriers are initially generated through a process of membrane budding and fission. They are then transported to the relevant acceptor membrane, with which they undergo fusion. Numerous proteins and lipids are involved in the induction and regulation of each of these steps, with the former including the coat-, fission-, and tethering- and docking- proteins.[18-20] The fission and fusion are processes in which membrane lipids play a key role in controlling the shape of the membrane and in assisting in the merging of the leaflets of the bilayers.[21] Membrane fission can to a certain extent be considered as the opposite of membrane fusion, where the membrane leaflets involved are external to the plasma membrane (or lumenal, within the cell) rather than being the cytosolic leaflets (see scheme in Fig. 2). However, these different topologies imply large physical differences: (1) cytosolic factors can only act on the leaflet opposite to the ones that initially interact and hemifuse (fusion of a single leaflet of a bilayer); (2) the close membrane contact that is the first event of the process requires membrane bending and the formation of highly constricted neck.[21]

Lipids, which are major components of the bilayer, are key determinants in the formation of the hemifusion intermediates that are probably generated during the fission and fusion of membranes. These biological lipids are classified on the basis of their molecular shape and structure, which determine how they interact and organise in an aqueous environment.[21] Conventionally, lipid molecules with an inverted cone-like shape induce a positive spontaneous curvature to the membrane, while the cone-shaped lipids give a negative spontaneous curvature. Here, the positive curvature of the membrane would correspond to its bending towards the cytoplasm; conversely, a negative curvature promotes the bending of the membrane towards the lumen or the extracellular medium (Fig. 2A).

Thus, the proteins that control lipid metabolism, translocation and intrabilayer flip-flop (from one leaflet to the other) can also be key determinants in the processes of fission and

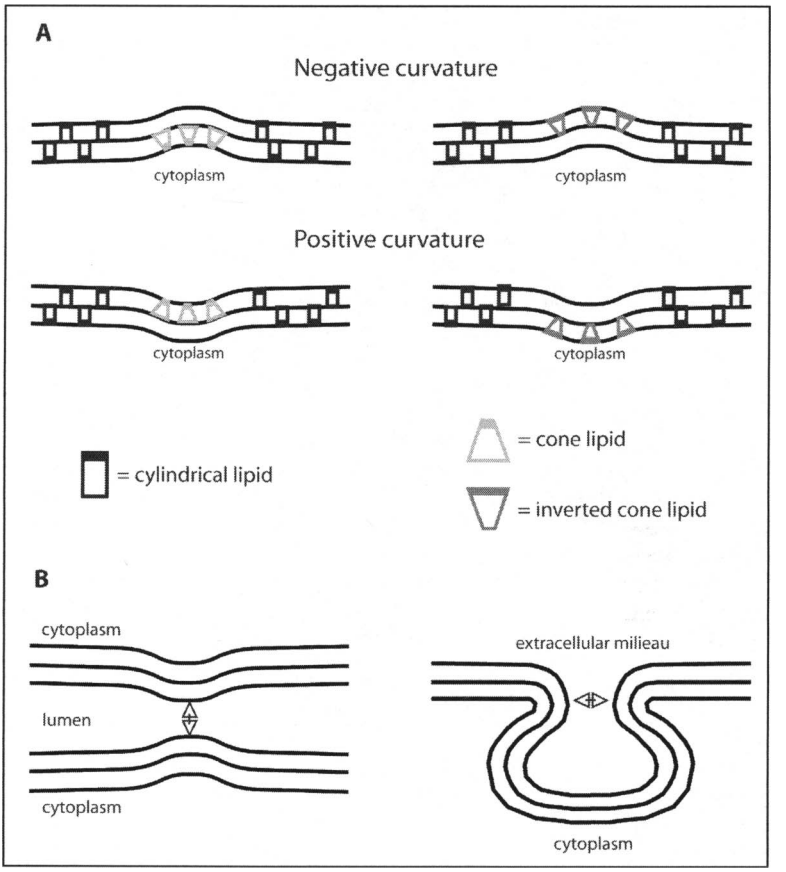

Figure 2. Spontaneous curvature of lipid bilayers. Panel A) schematic drawing of spontaneous membrane curvature and bending. When the cytoplasmic monolayer is enriched in cone-shaped lipids or the lumenal/external monolayer is enriched in inverted cone lipids, the bilayer acquires a negative spontaneous curvature and bends towards the organelle lumen or the outside of the cell. Conversely, when the cytoplasmic monolayer is enriched in inverted cone lipids or the lumenal external monolayer is enriched in cone-shaped lipids, the membrane acquires a positive spontaneous curvature and bends towards the cytoplasm. Panel B) diagrams showing the contact between bilayers during the membrane fission of intracellular tubules (left) or at the plasma membrane (right).

fusion. Examples of this category of proteins are the phosphoinositide transfer proteins (PITPs), which have been proposed to control the formation of transport carriers from the *trans*-Golgi network (TGN). This process appears to operate through the delivery of phosphatidylinositol (and, later, its phosphorylated derivatives) to specific sites on the TGN membranes, thus increasing the levels of the specific polyphosphoinositides that are required for fission.[22,23]

The Role of CtBP3/BARS in Membrane Fission

An analysis of the effects of recombinant or purified rat brain CtBP3/BARS on isolated Golgi membranes allowed us to begin to understand its action on the Golgi complex.[17] This was achieved by adding CtBP3/BARS to isolated Golgi membranes that were visualised by both conventional electron microscopy (EM) (resin embedding and thin sectioning) and by negative-staining EM.[17] In vivo, the Golgi ribbon is composed of the compact zone of stacked

cisternae (flat disc-like membrane "containers"), and the non-compact zone of tubular membranes. When these Golgi membranes were isolated and exposed to either recombinant or partially purified rat brain CtBP3/BARS in the presence of rat brain cytosol, the tubular structures underwent a rapid disruption, resulting in the formation of vesicular fragments of irregular sizes.[17] This fission/fragmentation was inhibited when the cytosol was immuno-depleted of CtBP3/BARS and was regained when this depleted cytosol was supplemented with the recombinant or partially purified CtBP3/BARS. A feature of these Golgi membranes in the presence of CtBP3/BARS-enriched cytosol was the early appearance of a number of sites along tubules where their diameter was greatly reduced. Sometimes several such constrictions were present along the same tubule, with a spacing at regular intervals (85 ± 3 nm). These dimensions are thus compatible with those of vesicles produced by membrane fission.[17] For this reason, it was proposed that these represent sites where fission will occur, and hence these constricted tubules were referred to as "fission intermediates".[17]

To investigate the mechanisms of this CtBP3/BARS-dependent formation of fission intermediates, the activity of CtBP3/BARS was assayed in the absence of cytosol, but with the readdition of different cytosolic factors. Strikingly, palmitoyl-CoA was able to regenerate the fission-inducing activity of the cytosol in the presence of purified or recombinant CtBP3/BARS.[17] This indicated that CtBP3/BARS might be involved in acyl-CoA-dependent lipid modifications. This hypothesis was tested by incubating a series of lipids and lysolipids, and their corresponding headgroups, with recombinant CtBP3/BARS and radiolabelled palmitoyl-CoA. Lysophosphatidic acid (LPA) was the only lipid that showed a detectable activity as an acyl acceptor from the [^{14}C]-palmitoyl-CoA, which resulted in the incorporation of the label into a new radioactive species, phosphatidic acid (PA).[17] Thus, CtBP3/BARS has an acyltransferase activity with a substrate selectivity for LPA. To be functionally relevant, this transferase activity of CtBP3/BARS had to be able to change the PA levels in Golgi membranes under the conditions used in the fission assay above. Therefore, the CtBP3/BARS-induced increase in PA levels and the fission of the Golgi membranes were compared in parallel. This showed that these two processes occurred within the same time interval, suggesting that they were indeed associated. These and other data[17] led to the proposal that the LPA acyltransferase activity of CtBP3/BARS is an essential component of the mechanism by which this protein promotes fission in Golgi tubular networks. Similarly, endophilin, a protein that does not share any significant homology with CtBP3/BARS, also induces fission at the plasma membrane by catalysing the conversion of LPA into PA.[24] This demonstration that an acyltransferase activity of two independent proteins is involved in membrane fission has thus further indicated the relevance of lipids and their specific composition in this process.[18,21,24] However, it should be pointed out that the acyltransferase activity associated to both CtBP3/BARS and endophilin is very slow.[17,24] This raises the question as to whether this activity is sufficient per se to support fission. In the case of CtBP3/BARS-dependent mitotic Golgi fragmentation (see below), this has been shown not to be the case.[25] A detailed molecular definition of the CtBP3/BARS-dependent fissioning machinery will certainly help to determine the contribution that this acyltransferase activity has in the fissioning process in different transport steps. An alternative, hypothetical model proposes that the changes in membrane geometry required for fissioning to occur, are induced by a conformational switch of the protein involved (either CtBP3/BARS or endophilin), which is determined by the specific lipid bound to it.[26] In this case, the acyltransferase-driven changes between the LPA-, acyl-CoA-, or PA-bound forms would affect the conformation and orientation of the protein in the membrane, thus leading to fission.[26]

CtBP3/BARS in Mitotic Fragmentation of the Golgi Complex

The fissioning of Golgi membranes is an important event also during cell division, when the Golgi complex undergoes an extensive fragmentation.[27] This process is thought to facilitate the equal partitioning of Golgi membranes between the two daughter cells. Although there remains an open debate about the final fate of the mitotic fragments of the Golgi complex, there is a

general agreement about the sequential fragmentation processes of the Golgi membranes. These begin in prophase and initially involve the severing of tubules that connect adjacent stacks.[28] During late prophase/prometaphase, the isolated stacks are shortened and transformed into a tubular network that is fragmented into clusters of vesicles and tubules.[28] This fragmentation process finally leads to a dispersion in the cytoplasm of the Golgi membranes, which have been proposed to either redistribute into the ER[29,30] or remain as isolated vesicular-tubular clusters.[27]

CtBP3/BARS has recently been shown to be an essential component of the machinery controlling the mitotic fragmentation of the Golgi complex.[25] The involvement of CtBP3/BARS was assessed by using a well-established assay that uses permeabilised normal rat kidney (NRK) cells in an incubation with mitotic cytosol (to mimic mitotic fragmentation of the Golgi in vitro[31]). This results in the break-up of the Golgi ribbon into tubular-reticular clusters, which then disperse throughout the cell, thus reproducing the fragmentation of the Golgi complex observed during mitosis.[32] From a morphological point of view, these fragments are similar to the Golgi clusters that are characteristic of prometaphase in intact cells.[32]

The requirement for CtBP3/BARS in this mitotic fragmentation emerged from experiments employing mitotic extracts that were either immuno-depleted of CtBP3/BARS or added with dominant-negative mutants of this protein (SBD and NBD, see also below[33]).[25] CtBP3/BARS depletion inhibited Golgi fragmentation by more than 80%, as did the addition of SBD and NBD (by 55% and 75%, respectively). Since the addition of recombinant CtBP3/BARS to depleted extracts, or together with SBD or NBD in mitotic cytosol, completely restored the fragmentation activity, these data were taken as a direct demonstration of the requirement of this protein in the fragmentation of the Golgi complex in the permeabilised cell system.[25]

Information on the mechanism of action of CtBP3/BARS was obtained by analysing the ultrastructure of the Golgi complex in these permeabilised cells by EM. Analysis of serial sections showed that in cells exposed to mitotic cytosol the Golgi complex was fragmented into small tubulo-vesicular elements (~0.5 μm in size) that were dispersed throughout the cytoplasm, thus also confirming previous immunofluorescence and EM data.[32] In contrast, when inhibitors of CtBP3/BARS, such as NBD and the p50-2 anti-CtBP3/BARS antibody, were included with the mitotic cytosol, the Golgi complex appeared to be still localised in the perinuclear area. This was in agreement with immunofluorescence observations,[25] even if the characteristic stacked organization of the Golgi complex was transformed into groups of large tubular-vesicular-saccular networks (~1.2 μm in size).[25]

The scheme of mitotic fragmentation that emerges from these studies envisions two separate steps that can also be identified as a CtBP3/BARS-independent and a CtBP3/BARS-dependent process.[25] During the former, which can be brought about by the inhibition of CtBP3/BARS activity, the Golgi stacks tubulate, possibly due to the phosphorylation of Golgi matrix protein such as GM130 and GRASP65, a modification known to occur during mitosis.[27,34,35] During the latter, the fissioning of the tubulated Golgi membranes occurs, by a mechanism requiring CtBP3/BARS activity[25] (Fig. 3).

This model has been further substantiated by analysing isolated Golgi membranes by negative staining and EM.[25] Exposure to mitotic cytosol completely disrupted the isolated Golgi membranes that appeared to be transformed into clusters of vesicles of various sizes and tubules presenting constrictions that were previously defined as fission sites.[17,25] Similar to the observations in permeabilised cells, the Golgi membranes appeared highly tubulated and without signs of fragmentation when the added mitotic cytosol was depleted of CtBP3/BARS. The addition of recombinant CtBP3/BARS to this depleted extracts completely restored the fragmentation activity of the mitotic cytosol.[25] Altogether, these observations are in line with the role of CtBP3/BARS as the fissioning protein that works at the level of the Golgi tubules in vitro[17] and during mitotic fragmentation[25] (Fig. 3).

This functional role of CtBP3/BARS was also analysed in living cells, by using two different approaches to inhibit the endogenous protein. One was the microinjection of CtBP3/BARS inhibitors (including the p50-2 anti-CtBP3/BARS antibody and SBD) in synchronized NRK

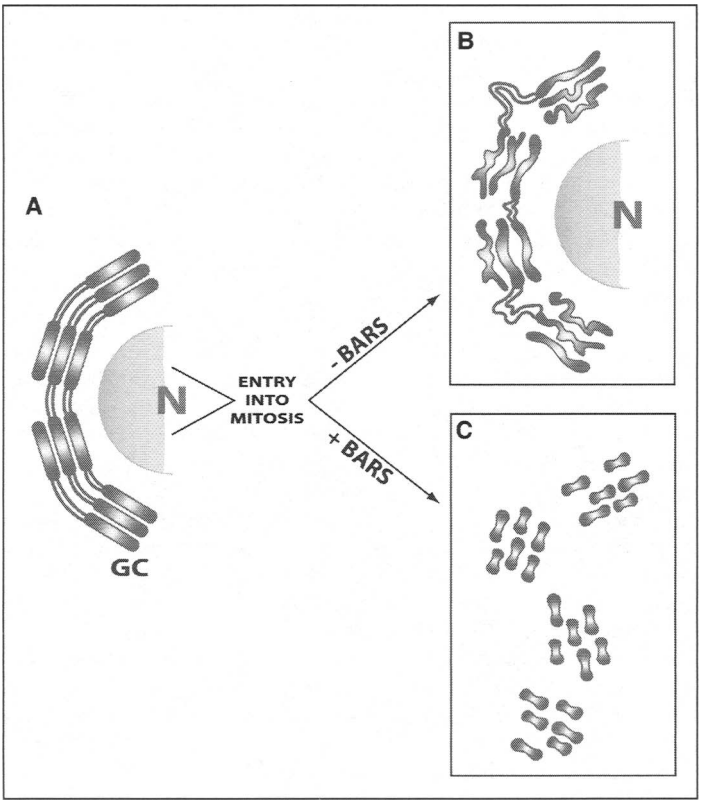

Figure 3. Schematic of the fragmentation of the Golgi complex during mitosis. A) The intact Golgi complex (GC) composed of interconnected stacks of cisternae that form a ribbon that encircles the nucleus (N), during interphase. B) Tubulation of the Golgi membranes at late G2/early prophase is observed in the absence of CtBP3/BARS. C) The Golgi complex at the beginning of mitosis, where the tubules connecting the different stacks and the cisternae undergo fission with a mechanism that depends on CtBP3/BARS. At this point, the fragments that form are dispersed into the cytoplasm.

cells that had been allowed to enter mitosis after releasing the block in the S phase of the cell cycle (obtained by aphydicolin treatment[25]). Indeed, after microinjection, these cells were not able to enter mitosis normally and the percentage of mitotic cells was decreased by 75%.[25] If CtBP3/BARS was microinjected together with SBD, the fragmentation of the Golgi complex was restored, and the cells progressed normally into mitosis. This, thus supported the crucial role of this CtBP3/BARS-dependent fragmentation for cell cycle progression.

If Golgi fragmentation is the crucial step for cell entry into mitosis and this can be inhibited by blocking the fragmenting/fissioning protein CtBP3/BARS, this block might be overcome by inducing a similar fragmentation by pharmacological agents that are able to disrupt the Golgi membranes by different mechanisms. This hypothesis was shown to be correct, and indeed, CtBP3/BARS-inhibited cells progressed into mitosis when the Golgi complex was artificially fragmented by drugs such as nocodazole, which disperses the Golgi ribbon into ministacks,[36] or BFA, which disassembles the Golgi membranes.[7,8]

The second approach used consisted in the treatment of NRK cells with antisense CtBP3/BARS oligonucleotides. Also in this case cells did not progress into mitosis (70% inhibition, as compared to scrambled-oligonucleotide-treated cells) and interestingly, they were arrested in

G2, as shown by specific markers of the cell cycle.[25] These cells resumed progression into mitosis after microinjection of recombinant CtBP3/BARS as well as after the forced dispersion of the Golgi membranes with nocodazole or BFA.[25]

Altogether these studies indicated that: (a) CtBP3/BARS has a key role in the fission of Golgi membranes during mitotic fragmentation; and (b) CtBP3/BARS-induced Golgi fission is required for entry into mitosis. These data reinforce the concept that the fragmentation of the Golgi complex is monitored by a checkpoint for cell cycle progression.[37]

Aspects that remain to be elucidated include the specific mechanisms of CtBP3/BARS activation during mitosis and the identification of the component(s) of the BARS-dependent fissioning machinery. While a number of CtBP3/BARS interactors have been identified[38] their role in membrane fission is still under investigation.

Interestingly, one known enzymatic activity of CtBP3/BARS, i.e., the slow acyltransferase activity reported for both of the fissioning proteins CtBP3/BARS and endophilin,[17,24] does not appear to be crucial in the mitotic fragmentation of the Golgi complex, but rather to have a facilitative function.[25] Thus, a CtBP3/BARS point mutant that is unable to catalyse the acyltransferase reaction was still able to induce fission, although with a potency 10-fold lower than that of the wild-type CtBP3/BARS.[25]

Another aspect that is presently under investigation is the control of mitotic progression in embryonic cells that are knocked-out for CtBP3/BARS.[39] While a mouse knocked-out for both *ctbp1* and *ctbp2* (and thus lacking CtBP3/BARS) was embryonically lethal, fibroblasts derived from these embryos divided normally.[39] This would indicate that other mechanisms might have overcome the lack of the CtBP3/BARS fissioning machinery in these cells. Work is in progress in our laboratory to fully define this mechanism. Data obtained so far are consistent with the idea that the organization of the Golgi complex in these embryonic cells is not normal, and is such that it can indeed overcome the requirement for CtBP3/BARS-induced fissioning (manuscript in preparation). This "adaptation" of Golgi membrane organization is analogous to other types of adaptation events for different cell functions that occur during embryonic development, as has been discussed recently.[40]

CtBP3/BARS as a Dual-Function Protein: The Molecular Switch

How can such similar proteins like the CtBPs have such different cellular functions? Possible answers to this question came from two independent analyses of the crystal structure of human CtBP1[41] and rat CtBP3/BARS.[33] Although the results obtained relating to the structure of the protein were similar in these two studies, some contrasting conclusions were made following parallel mutagenesis and biochemical analyses. In both cases, the truncated proteins used covered more than 80% of their full sequences and they were both cocrystallized with NAD(H). The binding of the dinucleotide, which was not included in the crystallization solution, probably derives from a specific high-affinity interaction between the protein and the dinucleotide. Indeed, the interactions of CtBP with its transcriptional partners and corepression activity are regulated by the nicotinamide adeninine dinucleotides NAD^+ and NADH.[42] More recently the affinities of NADH and NAD for CtBP1 have been measured, with NADH having an affinity some 100-fold higher than NAD^+ for CtBP1.[43]

These two crystallographic studies both showed that these CtBP proteins can form stable dimers from two identical subunits. Each subunit is organized in two compact domains that are separated by a deep cleft.[33,41] Interestingly, resolution of the CtBP crystal structure also revealed strong structural similarity between CtBPs and the family of the D-stereoisomer-specific 2-hydroxyacid NAD-dehydrogenases,[44,45] and accordingly to the literature on this latter class of proteins, the two domains were referred to as nucleotide-binding (NBD, residues 113-308) and substrate-binding (SBD, residues 1-112, 309-350) domains.[33] Superimposition of the structures of 2-hydroxyacid dehydrogenases and CtBP3/BARS revealed that the latter assumes a "closed" conformation, where the substrate- and nucleotide-binding domains are in close

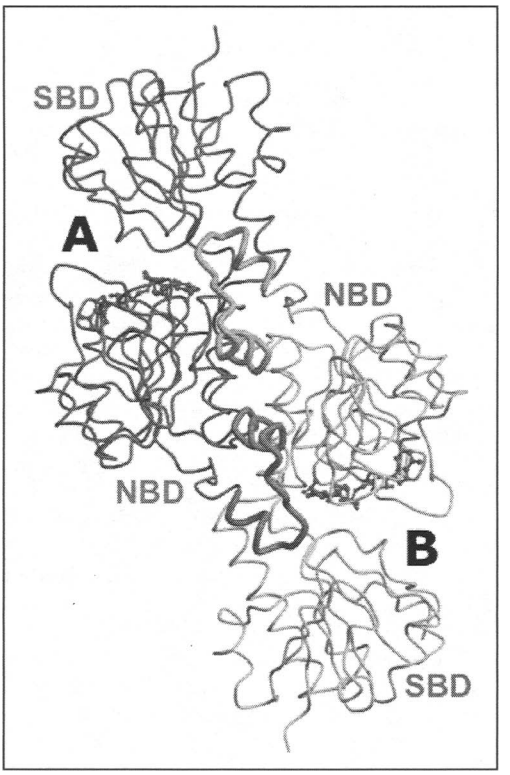

Figure 4. Structure of CtBP3/BARS (residues 1-350). A color version of this figure is available online at http://www.Eurekah.com. The two subunits of the dimer, A and B, are shown in different colors; red and green, respectively. The substrate-binding domain (SBD) and the nucleotide-binding domain (NBD) are indicated. The swapping domain (residues 132-154 of each subunit) is shown in blue, with the structural elements contributed by subunit B in a lighter shade of blue. The bound NAD(H) is shown in magenta.

contact, relative to the 2-hydroxyacid dehydrogenases. The CtBP3/BARS closed conformation is triggered by specific interactions at the dimerization interface, where both subunits contribute helices to form a swapping domain (Fig. 4). Nardini et al suggested that NAD(H) binding to CtBP3/BARS is required to promote the stabilization of the closed form of the protein observed in the crystals.[33] Attainment of this closed form is required to promote stabilization of a tight protein dimer through the domain-swapping mechanism.

Structural and biochemical analyses indicated that the protein has the ability to bind acylCoA in the same pocket where NAD(H) was revealed to be bound in the crystallized protein.[33] Indeed, long chain acylCoAs binds to CtBP3/BARS with an affinity in the low micromolar range and both NADH and NAD are competitors of this binding. Moreover, based on the common ADP moiety shared by NAD(H) and CoA, a CoA molecule was modelled in the interdomain cleft at the NAD(H) site. This structural modelling resulted in a good match of the common molecular structures.[33] However, in the closed CtBP3/BARS conformation there is no room available to accommodate the long aliphatic tail of the acyl-CoA due to the presence of two α-helices contributed by the opposing dimer subunits. Indeed, the binding of an acyl-CoA molecule with a [^{14}C]-C20 aliphatic tail (needed for fission activity[17]), was predicted to perturb the domain-swapping interaction, that stabilizes CtBP3/BARS dimer association. Thus, it

was proposed that the binding of long chain acyl-CoA must be coupled with, and therefore induce, a more open conformation of CtBP3/BARS, similar to that adopted by NAD-dependent dehydrogenases.[33]

In addition, the LPA-acyltransferase activity of CtBP3/BARS requires that LPA binds to the protein and approaches to the bound acyl-CoA. An open form of the protein would be required for a substrate as large as LPA to approach the bound acyl-CoA. Interestingly, a protein tunnel connecting the NAD(H)/acyl-CoA binding site to the solvent in CtBP3/BARS is partly detectable in the closed enzyme form, and is lined mainly with positively charged residues, which would contribute the correct electrostatic environment for the accommodation of the negatively charged LPA head group.[33] These crystallographic studies thus led to the proposal that LPA would be able to approach the cofactor binding site only in the acyl-CoA-bound form of CtBP3/BARS, as the substrate and nucleotide-binding domains move apart. This implies that the two activities of the CtBPs, the nuclear corepression activity and the Golgi membrane fissioning activity, may be regulated by the availability of the two cofactors, NAD(H) and acyl-CoA.[33] In particular, at the level of the Golgi complex, the acyl-CoA concentration could be higher or the availability of acyl-CoA could be increased by the presence of acyl-CoA binding proteins; this will result in an acyl-CoA-bound CtBP3/BARS exhibiting acyl transferase activity. Additional studies are required to further analyze this hypothesis and to unveil the mechanism of the switch between the two cellular functions of the CtBPs.

Altogether, however, the above data suggest that the alternative binding of NAD, NAD(H) and acyl-CoA to the CtBP proteins determines not only their conformation but also their cellular function (regulation of transcription or of membrane fission).

An aspect remaining unsolved is whether the three members of the CtBP family each serve a specific role in the cell, or whether they act at both the nuclear and cytosolic levels. As reported, CtBP1 and CtBP2 are transcriptional corepressors that also have roles in development and oncogenesis.[46] CtBP3/BARS has been reported to have a role in membrane fission,[17,18,25] (see above). Due to their high degree of homology, it is conceivable that these CtBP proteins could interchangably modulate fission as well as transcription. If this is true, the CtBPs would join the several already reported cases of proteins that are endowed with dual functions, one in the nucleus and another in the cytoplasm.[47-50] One should thus address the questions as to whether there is a common regulation of such different cellular functions, if they can be driven by the same protein, and what regulates the functional switch. Data already available indicate that posttranslational modifications (or binding to cofactors) of proteins can determine their cellular localization. Thus, phosphorylation of CtBP1 by PAK1 has been associated to its translocation from the nucleus to the cytoplasm, and to the inhibition of its transcriptional activity,[51] whereas an opposite effect has been reported following its sumoylation.[52] The mutually exclusive binding of NAD(H) or acyl-CoA to CtBP3/BARS could represent another case of localization and functional switches being controlled by protein modification.

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CHAPTER 11

CtBPs as Synaptic Proteins

Susanne tom Dieck, Frank Schmitz and Johann Helmut Brandstätter*

Summary

A surprising new aspect of CtBP family proteins arose from the identification of a novel CtBP protein named RIBEYE.[1] RIBEYE, which consists of a unique amino-terminal A-domain and a carboxy-terminal B-domain, largely identical to CtBP2, was discovered not as a nuclear protein but as a major component of synaptic ribbons in mammalian retina.[1] Ribbon synapses are structurally specialized, tonically active chemical synapses, and are present, for example, in the sensory neurons of the retina and the inner ear.[2,3] Recently, we identified also CtBP1, the founder member of the CtBP family,[4] as an active zone component at conventional and ribbon synapses.[5] The discovery of synaptic CtBP family members highlights that CtBP proteins serve more functions than previously envisioned.

Chemical Synapses and Synaptic Transmission

Chemical synapses are highly complex contact sites between neurons specialized for the rapid and efficient transmission of synaptic signals. Ultrastructurally, distinct pre and postsynaptic regions mark the sites of neurotransmitter release and reception (Fig. 1A). In the synaptic terminals, neurotransmitter-filled synaptic vesicles translocate to a specialized region of the presynaptic plasma membrane, the active zone. Here they undergo an ATP-dependent priming step that makes them releasable by exocytosis. Activity-triggered Ca^{2+} influx through voltage-gated Ca^{2+} channels triggers fusion of the primed synaptic vesicles with the plasma membrane and subsequent neurotransmitter release into the synaptic cleft. After exocytosis, the synaptic vesicle membrane is rapidly retrieved by endocytosis, refilled with neurotransmitter and recycled for a new round of the synaptic vesicle cycle.[6] A specialized cytomatrix at the active zone spatially organizes these events in the presynaptic terminal. This cytomatrix at the active zone (CAZ) is an electron-dense cytoskeletal meshwork, which extends into the synaptic terminal where it associates with synaptic vesicles.[7] The mature CAZ is defined by a set of multidomain proteins that harbor several protein-protein or protein-lipid interaction domains. It includes proteins like Munc13-1,[8] RIMs,[9,10] ERC/CAST,[11,12] Piccolo/Aczonin and Bassoon.[13-15] The complete protein composition of the CAZ is not known to date, as it is not known how the CAZ organizes the synaptic vesicle cycle.

Ribbon Synapses

Photoreceptors and bipolar cells in the retina and hair cells in the cochlea transmit light and sound signals, respectively, over a dynamic range of several orders of magnitude in intensity. They continuously adjust their synaptic output to changing inputs thus, optimizing the information transfer. Such a finely graded synaptic output requires the release of several

*Corresponding Author: Johann Helmut Brandstätter—Institute for Biology, University of Erlangen-Nürnberg, D-91058 Erlangen, Germany. Email: jbrandst@biologie.uni-erlangen.de

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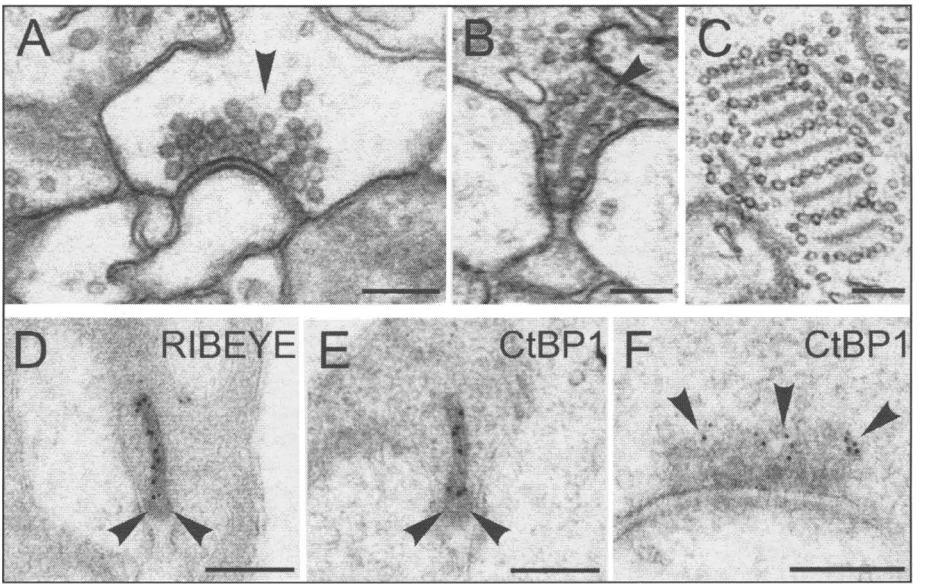

Figure 1. Photoreceptor and Amacrine Cell Synapses in the Retina, and the synaptic expression of RIBEYE and CtBP1. A) Electron micrograph of an amacrine cell synapse. The *arrowhead* points to the accumulation of neurotransmitter-filled synaptic vesicles at the presynaptic active zone. B) Electron micrograph of the ribbon synaptic complex in a rod photoreceptor terminal. The presynaptic ribbon (*arrowhead*) is anchored at its base in the arciform density, it is lined by a row of synaptic vesicles, and it faces two postsynaptic elements. C) In the Bassoon-deficient retina, the presynaptic ribbons, lined by synaptic vesicles, float freely in the cytoplasm of the photoreceptor terminals. D,E) Electron microscopy and postembedding immunogold labeling shows that photoreceptor ribbons are decorated with gold particles (10 nm) for RIBEYE (D) and for CtBP1 (E). Note the absence of gold particles at the base of the ribbons, the region of the arciform density (*arrowheads*). F) Electron micrograph of an amacrine cell synapse postembedding immunogold labeled for CtBP1. The gold particles for CtBP1 are located some distance away from the active zone at the edge of the electrondense CAZ material (*arrowheads*). Scale bars, 0.2 μm. (Fig. 1E,F reproduced from J Cell Biol 2005; 168:825-836, by copyright permission of The Rockefeller University Press.[5])

hundreds to several thousands of vesicles per second.[16] To accomplish this level of performance, these sensory neurons maintain large pools of readily releasable synaptic vesicles, and are equipped with a special type of chemical synapse, the ribbon synapse[17] (Fig. 1B). The presynaptic ribbon constitutes an electron-dense band of large surface area that extends from the site of transmitter release into the presynaptic cytoplasm and tethers hundreds of synaptic vesicles.[18] The synaptic ribbon was thought to be a unique structure specialized to ribbon synapses in sensory organs. An emerging idea, however, is that all chemical synapses are organized according to a common principle in which structural differences correlate with the kinetics of transmitter release.[7] Within this concept, every synapse has dense projections on which vesicles are tethered, and the ribbon is a variation of this common theme. A scaffold of proteins that are just beginning to be identified define and organize the ribbon. One of these proteins is RIBEYE.

The Novel CtBP Protein RIBEYE Is a Component of Synaptic Ribbons

When synaptic ribbons were purified biochemically,[19] a 120 kDa protein named RIBEYE was identified as an integral component unique to these structures[1] (Fig. 1D). Sequence analysis revealed that RIBEYE is a member of the CtBP family with an intriguing domain

structure. RIBEYE consists of a large and unique amino-terminal A-domain, encoded by a single exon located within a large intron, and a smaller carboxy-terminal B-domain that is identical to CtBP2 except for the first 20 amino acids. Thus, RIBEYE is a transcript variant of the CtBP2 gene.

CtBP1 Is Present at Both Ribbon and Conventional Chemical Synapses

It was tempting to speculate that the generation of two different gene products from the CtBP2 gene reflects two different roles, a synaptic and a nuclear one, dependent on the presence or absence of the A-domain. In a recent study, however, we showed that the situation is more complex. In addition to RIBEYE, CtBP1 was identified as a component of synaptic ribbons (Fig. 1E). Moreover, CtBP1 is present at conventional chemical synapses, which do not express RIBEYE[5] (Fig. 1F). Ultrastructurally RIBEYE and CtBP1 localize to the entire extension of the synaptic ribbon. The arciform density, the region adjacent to the active zone at the ribbon base, is devoid of the two proteins[1,5] (Fig. 1D,E). At conventional amacrine cell synapses, CtBP1 immunolabel marks the release site, but it is located some distance away from the active zone at the edge of the electron dense CAZ material[5] (Fig. 1F). This indicates that the CtBP domain as common denominator has both a nuclear and synaptic function, and that the novel variant RIBEYE has most likely developed to meet the high kinetic demands of tonically releasing ribbon synapses.

In-vivo labeling experiments with fluorescent peptides that bind RIBEYE and CtBPs showed approximately 4000 available peptide binding sites at synaptic ribbons of goldfish bipolar cells.[20] The large number is compatible with the idea that RIBEYE/CtBPs are main components of synaptic ribbons.

Diversity of *CtBP* Genes

CtBP1 and the RIBEYE B-domain/CtBP2 are highly homologous and conserved between species, whereas the A-domain is highly divergent (Fig. 2). Based on this finding, the CtBP domain seems to have a general function at synapses, and the A-domain seems to confer a ribbon-specific function. From the two mammalian CtBP genes, only the CtBP2 gene—on chromosome 10 in humans and on chromosome 7 in mice—contains an A-domain exon.[1,21] An even higher diversity in the *CtBP* family is seen in teleost fish,[22] consistent with the theory that the ancestral teleost underwent one round more genome duplication than did other vertebrate classes.[23,24] In zebrafish and fugu, two *RIBEYE* genes give rise to two different RIBEYE proteins, called RIBEYE a and RIBEYE b.[22] Both RIBEYE a and RIBEYE b contain a highly conserved B-domain, identical to CtBP2, and an A-domain, which is much less conserved (35% identity between zebrafish RIBEYE a and b). At least for the more abundant RIBEYE a protein a second splice variant was described with a cryptic splice site within the A-domain.[22]

Functional Considerations

Little is known about the role of CtBP proteins at chemical synapses. Based on the established functions of CtBP family members, we would like to propose two functional roles for synaptic CtBP proteins: a structural role, and a role in membrane turnover.

A Structural Role of CtBPs at Chemical Synapses

We have found, that both CtBP family members, CtBP1 and RIBEYE, interact directly with the presynaptic cytomatrix protein Bassoon. In a mouse mutant lacking the CtBP binding site in Bassoon, photoreceptor ribbons are not anchored at the active zone but float freely in the cytoplasm, and photoreceptor synaptic transmission is greatly impaired (Fig. 1C). Thus, the interaction between CtBPs and Bassoon is responsible for the physical and functional integrity of the ribbon synaptic complex of retinal photoreceptor cells.[5,25]

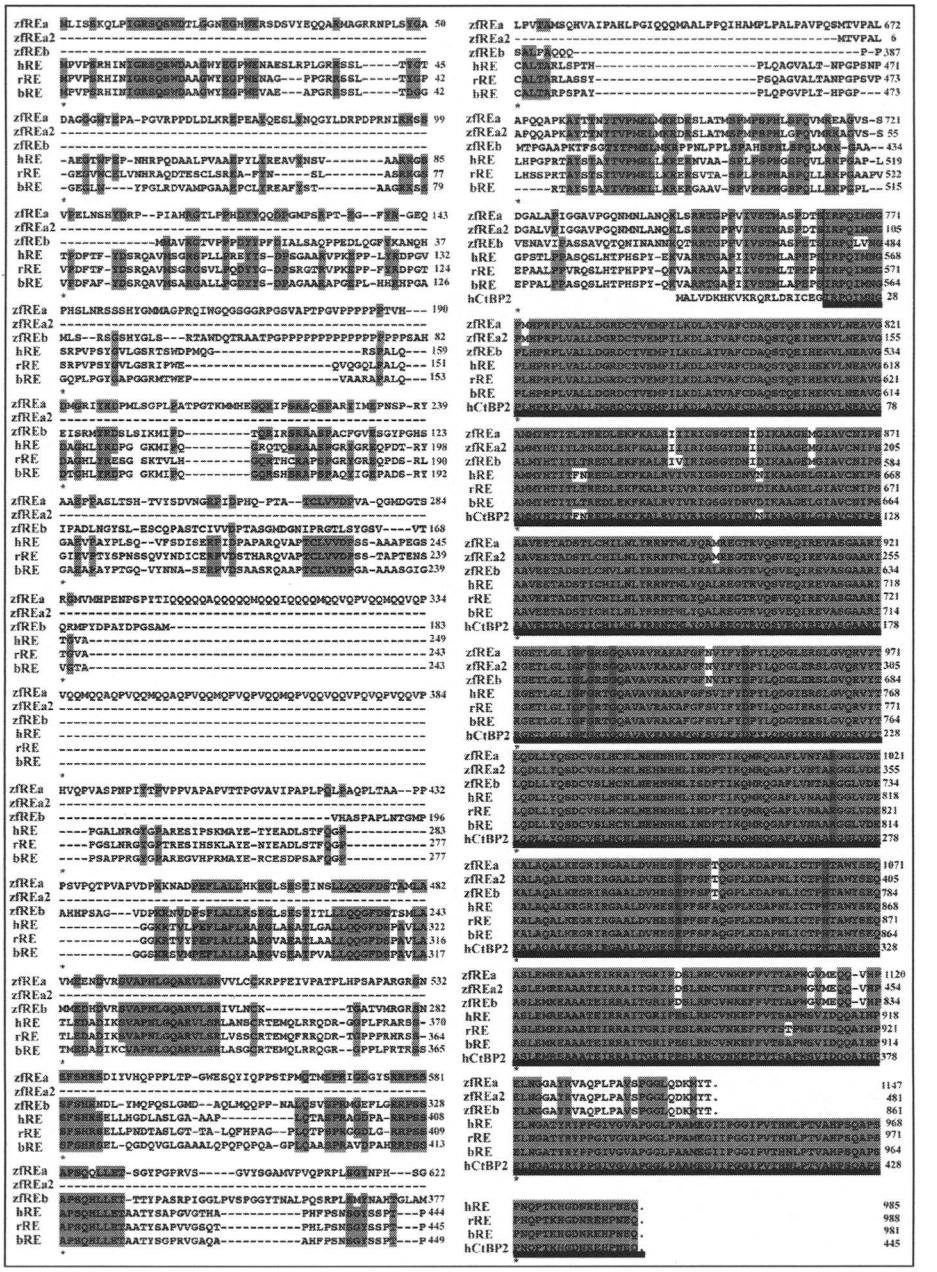

Figure 2. Protein sequence alignment of RIBEYE proteins from different species (zebrafish, human, rat and bovine). Amino acid identities are highlighted in red. Abbreviations: zfREa: zebrafish RIBEYE a; zfREa2: zebrafish RIBEYEa2; zfREb: zebrafish RIBEYE b; hRE: human RIBEYE; rRE: rat RIBEYE; bRE: bovine RIBEYE; hCtBP2: human CtBP2. The B-domain of RIBEYE, which is highly conserved between species, is underlined in black. Amino acids highlighted in blue are central components of the NAD^+-binding motive common to all RIBEYE/CtBP proteins. A color version of this figure is available online at http://www.Eurekah.com.

A structural role of RIBEYE has also been suggested based on the finding that the A-domain of RIBEYE forms protein clusters, which might create the ribbon backbone.[1] The clustering of RIBEYE molecules in the synaptic ribbon creates a high concentration of CtBPs at a limited plasma membrane area of the active zone. Interestingly, ribbon extension in photoreceptors varies in dependence of the light/dark cycle, thus linking ribbon length to the activity of the synapse.[26]

A strong indication for a structural role of RIBEYE came recently from a study by Wan and colleagues on zebrafish.[22] Morpholino knock-downs of *RIBEYE a* in zebrafish larvae inhibited synaptic ribbon assembly and led to the loss of the optokinetic response. Especially in bipolar cells where *RIBEYE a* seems to be the only *RIBEYE* gene expressed, ribbons with their characteristic pentalaminar structure were rarely found. Occasionally, unstructured accumulations of electron-dense material close to putative synaptic sites were observed.[22] Interestingly, in photoreceptor cells which contain both RIBEYE a and RIBEYE b, the attachment sites of ribbons with a small piece of ribbon material were still present. The authors suggested that this small residual ribbon complex might contain RIBEYE b.[22]

The *RIBEYE a* knock-down in zebrafish suggested also an unexpected developmental role of RIBEYE.[22] In RIBEYE a deficient zebrafish, bipolar cell development was arrested in a late stage, which inhibited the formation of large synaptic terminals and the expression of PKCα, and led to increased bipolar cell apoptosis. These events seemed to reverse when RIBEYE a expression recovered. One cannot say, however, whether RIBEYE has indeed a novel function in development or whether the observed defects are secondary due to lack of transmitter release caused by fault ribbons.

A Role of CtBPs in Membrane Turnover at Chemical Synapses

The transmitter-filled vesicles tethered to the synaptic ribbon constitute the readily releasable pool of synaptic vesicles at ribbon synapses.[2] Recently, a role of the ribbon in membrane turnover has been proposed by a study examining the release kinetics of inner hair cell ribbon synapses in the Bassoon mutant mouse.[27] In cochlear inner hair cells, like in retinal photoreceptors, anchoring of the synaptic ribbons is impaired in mutant mice deficient for Bassoon. This reduced the presynaptic readily releasable vesicle pool and caused a specific loss of the fast component of hair cell release; the sustained component of exocytosis remained.[27] Interestingly, floating ribbons were surrounded by tubular and cisternal membrane profiles that were only occasionally observed in the wild-type. This finding is reminiscent of the CtBP/BARS activity described in Golgi fissioning, which might be also relevant for the function of RIBEYE/CtBPs at the synapse.[28-30] RIBEYE/CtBP2 and CtBP1 display homology to NAD^+-dependent dehydrogenases and bind $NAD^+/NADH$.[1,31] In the context of preparing vesicles for exocytosis at the ribbon, they may function as lysophosphatidic acyl-CoA transferases and thus modulate the curvature of lipid membranes.[32]

Concluding Remarks

With the recent discoveries of proteins of the CtBP family at chemical synapses in the central nervous system, it becomes clear that CtBPs will serve more functions than previously envisioned. The characterization of synaptic CtBP functions, which is at its beginning, will greatly benefit from knock-out models. Unfortunately, no knock-out mouse is yet available for the *RIBEYE* gene to define its precise synaptic functions and its role during development in the mammalian retina. CtBP1 and CtBP2 knock-out mice have been generated.[33] Interestingly, in contrast to the CtBP2 knock-out mouse, the CtBP1 knock-out is viable, which indicates that CtBP2 can compensate for most of CtBP1's nuclear functions. Why the CtBP1 knock-out mice show a higher mortality than their wild-type siblings, remains to be clarified - maybe they die because of an uncompensated synaptic action of CtBP1.

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CHAPTER 12

A New Member of the CtBP/BARS Family from Plants: Angustifolia

Hirokazu Tsukaya*

Abstract

The ANGUSTIFOLIA (AN) gene in *Arabidopsis thaliana* (L.) Heynh. is the first homolog of the CtBP/BARS gene family identified in plants and is responsible for the polarity-dependent control of leaf cell expansion. This review compares the sequence homology and functional similarity of the AN protein with authentic animal CtBP/BARS family proteins. AN homologs have been found in both angiosperms and mosses, suggesting AN is conserved in terrestrial plant genomes. The AN subfamily is unique in having not only the D-isomer-specific 2-hydroxy acid dehydrogenase (D2-HDH) motif that is conserved among the CtBP/BARS family but also putative LxCxE/D and nuclear localization signal (NLS) motifs and a long C-terminal region. The absence of the catalytic triad, which is conserved in all D2-HDH sequences and is believed to be essential for the corepression activity of CtBP, suggests that AN might differ, at least in part, from CtBPs in molecular function. In addition, the distribution and density of the Golgi apparatus is normal in a null allele of the *an* mutant, suggesting that AN might not have a BARS function. An analysis of cytoskeletons in *an* mutant leaf cells suggests that *AN* might play an important role in controlling the arrangement of cortical microtubules that is plant-specific cytoskeletons. With all these attributes, AN appears to be the third member of an enigmatic family, CBA = CtBP/BARS/AN, which regulates aspects of developmental and organelle control in animals and plants.

Angustifolia—A Polarity-Dependent Regulator of Leaf Cell Expansion

Focusing on mechanisms that govern the polarized growth of leaves in the model plant *Arabidopsis thaliana* (L.) Heynh. (arabidopsis), we used mutational studies to identify two genes that act independently of each other to regulate polar cell elongation in leaves: *AN* regulates the width of leaves, and *ROT3* regulates the length.[1-4] The *angustifolia* (*an*) mutant of arabidopsis (Fig. 1) was originally isolated by Rédei.[5] The mutation in the leaf-specific *an* phenotype is caused by a specific defect in the elongation of leaf cells in the transverse (width) direction[1,2](Fig. 1A,C). This polar defect was observed in all the leaf cells examined, including epidermal cells, trichomes, and parenchymatous cells (Fig. 1B-D). The altered direction of cell elongation was particularly evident in palisade cells, where expansion in the leaf-width direction

*Corresponding Author: Hirozaku Tsukaya—Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology, 38 Nishigounaka, Myodaiji-cho, Okazaki 444-8585, Japan Graduate University for Advanced Studies, Shonan Village, Hayama, Kanagawa 240-0193, Japan; Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan. Email: tsukaya@nibb.ac.jp

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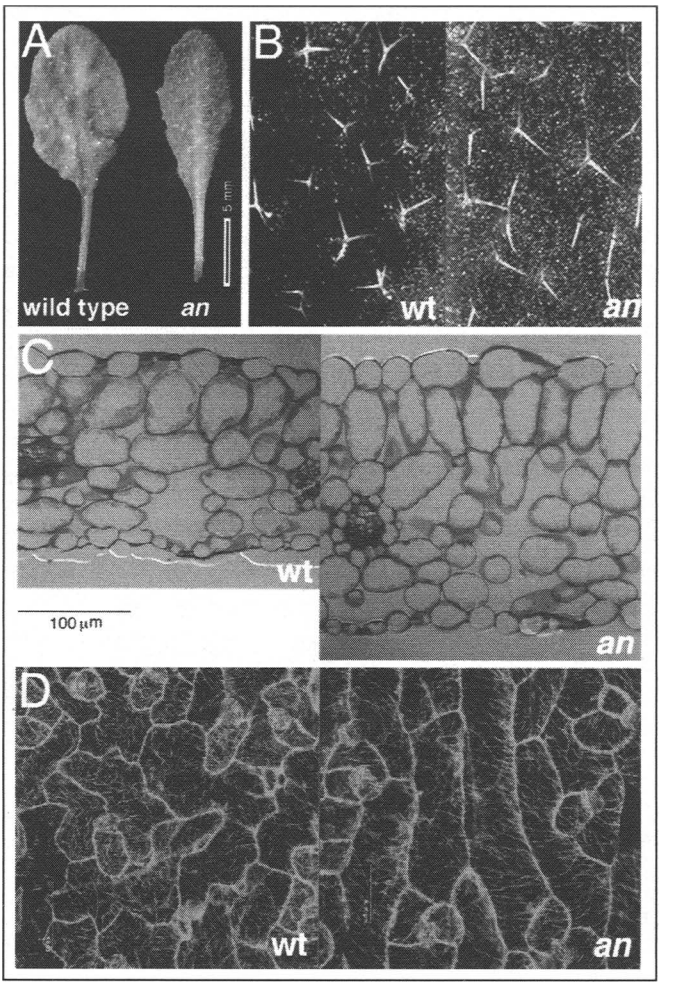

Figure 1. Morphological phenotypes of the *angustifolia* (*an*) mutant of arabidopsis. A) Gross morphology of leaves of the wild type and *an* mutant. Note the narrow shape of the *an* leaf. Bar, 5 mm. B) Trichomes on the leaf. Wild-type trichomes are three-branched in most cases (left), whereas *an* trichomes are two-branched. The longitudinal direction of the panel corresponds to the leaf-length direction. C) Cross-section of leaves of the wild type (left) and *an* mutant (right). Note the narrow, longer shape (in the leaf-thickness direction) of the *an* leaf cells. Bar, 100 μm. D) Arrangement of cortical microtubules (MTs) in leaf epidermal cells. The longitudinal direction of the panel corresponds to the leaf-length direction. Compared with wild-type MTs, the *an* MTs are arranged more simply and parallel to the leaf-width direction. Modified from Kim et al.[6]

was decreased, while elongation in the leaf-thickness direction was increased (Fig. 1C). Coincident with the defect in the palisade cells, the number of protrusions in epidermal cells was decreased, particularly in the leaf-width direction (Fig. 1D). A decreased number of branchings in trichome cells is also attributable to the same defect (Fig. 1B). Thus, the *AN* gene is thought to be the key gene to regulating the polar elongation of leaf cells in the leaf-width direction.[2] Cytological analysis showed that the *an* mutant has abnormally arranged cortical microtubules in leaf cells (Fig. 1D),[6,7] suggesting that *AN* might regulate polarity-dependent elongation of leaf cells via control of the arrangement of cortical microtubules.[6]

Recently we cloned the ANGUSTIFOLIA (AN) gene, a new member of the CtBP/BARS family from arabidopsis.[6,7] Microarray analysis suggested that the AN gene might function as a transcriptional corepressor, like the CtBPs.[6] Despite such speculation, the molecular function of AN is still unclear. Does AN regulate the arrangement of cortical microtubules by functioning as a plant CtBP? AN sequence data shows that the AN gene contains not only the D-isomer-specific 2-hydroxy acid dehydrogenase (D2-HDH) motif that is conserved among CtBP/BARS but also a putative LxCxE/D motif, which may be responsible for binding to retinoblastoma (Rb) protein,[8-10] the PEST motifs that are thought to be responsible for degradation,[11] a putative phosphorylation site for casein kinase II, and a nuclear localization signal (NLS; Fig. 2). A long C-terminal region is also unique to AN (Fig. 2). We previously reported that the *an-1* mutant allele has a nucleotide insertion in the C-terminus and that the *an-2* mutant allele has a nucleotide transition resulting in the introduction of a termination codon in the conserved D2-HDH motif.[6] Moreover, all known members of the AN subfamily have long C-terminal domains (Fig. 2). These data suggest that the C-terminal region might have an important role in the function of AN. More detailed comparative studies are required to determine whether the C-terminal region really superfluous to AN functions or not. Given that CtBP and BARS have similar amino acid sequences yet are functionally different, it is plausible that AN, although very closely related to CtBP/BARS, might differ from both in molecular function. This review compares AN protein with authentic CtBP family proteins.

Is AN a CtBP-Like Corepressor?

If AN is a corepressor like CtBPs, then transcription from certain genes should be up-regulated in the *an* mutant. We carried out microarray analysis between wild-type and *an-1* mutant plants under the auspices of the Monsanto *Arabidopsis* Microarray Program[6] and found that some genes were up-regulated in the *an* mutant but down-regulated in wild-type plants. RT-PCR analysis among wild type, *an-1* mutant, and transgenic *an* plants that expressed the wild-type *AN* gene confirmed the results. In particular, the expression of genes in the xyloglucan endotransglucosylase/hydrolase (XTH) family showed characteristic patterns among the plants. Quantitative, real-time RT-PCR showed that one of the *XTH* genes, *MERI5,* was expressed at a three-fold higher level in rosette leaves of the *an-1* mutant than in the wild type, while two other XTH genes, *EXGT-A1* and *EXGT-A2,* showed no differences in expression.[6] These data appear to support the idea that AN can act as a corepressor of a particular set of genes in arabidopsis. However, we should note that the double mutant *an-1/meri5* does not differ in morphology from the *an-1* single mutant (Yokoyama, R., personal commun). This suggests that the down-regulation of *MERI5* mRNA is not essential for the function of *AN.*

Does AN Act as a Molecular Bridge as CtBPs?

The CtBP of drosophila (dCtBP) and mouse (mCtBP2) self-associate in a yeast two-hybrid system,[12,13] and it has been suggested that the dimerization of CtBP is important for its molecular function.[14] Dimerized CtBPs are believed to function as a molecular bridge between a DNA-binding protein and a transcriptional repressor. We previously showed that AN self-associates as do CtBPs.[6] Does AN act as a molecular bridge as does authentic CtBP? The CtBPs have an intrinsic dehydrogenase activity,[15,16] and the NAD^+-dependent conformational change is thought to be essential to the corepression activity of CtBP.[15] In animal CtBPs, NAD^+-dependent dehydrogenase activity is also thought to be linked to the regulation of protein-protein interactions via the PxDLS recognition motif.[15] On the other hand, the AN gene subfamily is distinguishable from the CtBPs by its lack of the catalytic triad (His315/Glu1295/Arg266) conserved in all D2-HDH[15] (Fig. 2, shown by asterisks). A mutant CtBP in which His315 was changed to a valine lacks detectable dehydrogenase activity.[16] Thus, AN is expected also to lack dehydrogenase activity. In light of the above observation, AN appears to be unable to associate with the PxDLS motif. Moreover, AN does not have a GxGxxG(17x)D motif (Fig. 2), and a mutation of the GxGxxG(17x)D motif of CtBP has been shown to strongly

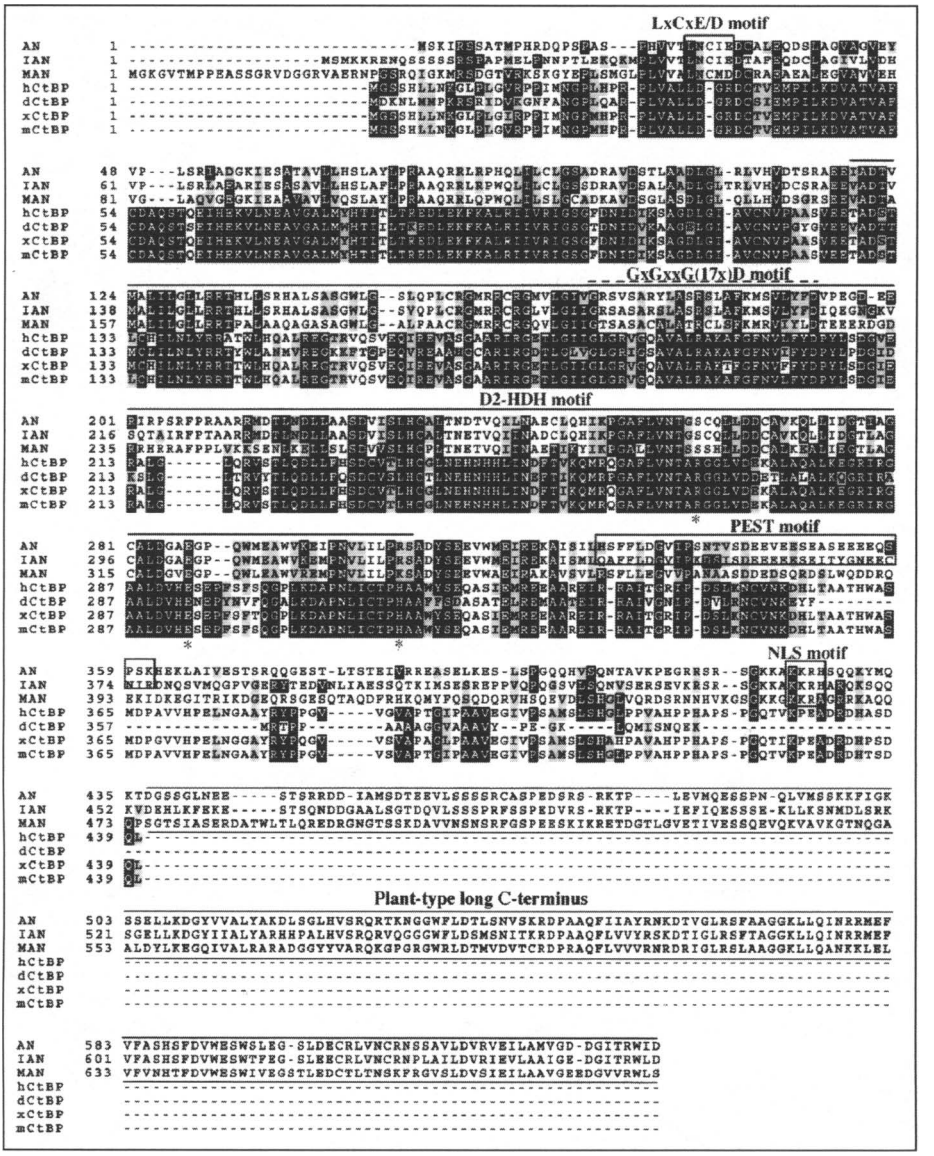

Figure 2. AN, AN homologs, and CtBPs. The alignment shows a comparison between the amino acid sequences of arabidopsis AN (AN from *Arabidopsis thaliana*, SWISS-PROT Q948X7), two plant AN homologs (IAN from *Ipomoea nil*, SWISS-PROT Q84JM5; MAN from *Marchantia polymorpha*, Q7XAP0), and several animal CtBPs (hCtBP from *Homo sapiens*, SWISS-PROT Q13363; dCtBP from *Drosophila melanogaster*, SWISS-PORT O46036; xCtBP from *Xenopus laevis*, SWISS-PORT Q9YHU0; and mCtBP from *Mus musculus*, SWISS-PROT O88712). All sequence alignments were performed by Dr. K.-H. Cho (Dong-A University, Korea) using the CLUSTAL W program.[24] The asterisks indicate the dehydrogenase catalytic triad of D2-HDH. See text for further details.

inhibit the ability of CtBP to bind the PxDLS motif.[15] To determine whether AN interacts with E1A, we carried out a yeast two-hybrid analysis using the C-terminal region of E1A as bait

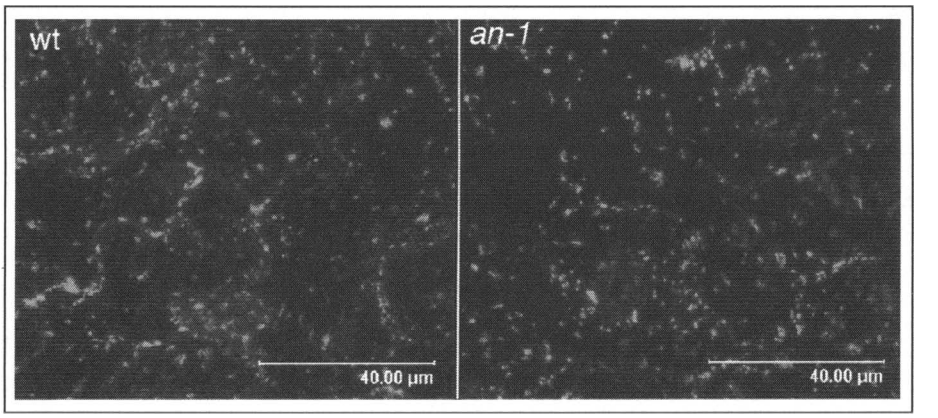

Figure 3. Golgi apparatus of wild-type and *an* mutant leaf cells. The Golgi apparatus in leaf epidermal cells was visualized by the Golgi-specific GFP marker *AtErd2::GFP*.[25] Bar, 40 µm. Note that there are no apparent differences in the size and density of the Golgi apparatus between the wild-type (left) and the *an* mutant (right).

and the full-length AN as prey. The results indicated that AN does not interact with the E1A protein, which contains the PxDLS consensus recognition motif for animal CtBPs. (K.-H. Cho, G.-T. Kim, and H. Tsukaya, unpublished result), Thus, we conclude that AN cannot associate with proteins harboring the PxDLS motif.

Is AN a Homolog of BARS?

If AN is a functional homolog of CtBP, it should be localized in cell nuclei. Alternatively, if AN is a functional homolog of BARS, a member of the CtBP/BARS family that acts in the cytoplasm, an AN mutation might disrupt the Golgi apparatus, as BARS is essential for its establishment and maintenance.[17] It is possible that AN functions as CtBP in the nucleus and as BARS in the cytoplasm. However, no abnormality in the shape or number of Golgi stacks was observed in the *an-1* mutant leaf cells (Fig. 3). On the other hand, reports on the intracellular localization of AN[6,7] are contradictory. Folkers et al[7] reported that AN is localized in the cytoplasm and not in the nucleus, while our data showed that AN is detected in both nuclei and cytoplasm.[6] Our recent observations on stable transgenic arabidopsis harboring *ANp::AN::GUS* showed that both reports might be correct; we found that the localization of AN is developmentally changed (G. Horiguchi and H. Tsukaya, unpublished observation). Further analysis on the relationship between intracellular localization and the function of AN will supply us important clues on the role of AN in plant development.

Conserved Function in Plants

As stated above, understanding the role and importance of AN-specific motifs requires detailed analyses of AN homologs from different plant species. Most AN-specific motifs are conserved in members of AN subfamily. For example, AN and IAN, an AN homolog from Japanese morning glory (*Ipomoea nil*), have the LxCxE motif in the N-terminal region,[6,18] and MAN, an AN homolog from liverwort (*Marchantia polymorpha*), has a slightly different motif, LxCxD (Fig. 1). The PEST motif is conserved in IAN,[18] but poorly conserved in MAN, as defined by PESTFIND (http://bioweb.pasteur.fr/seqanal/interfaces/Pestfind-simple.html). In the case of MAN, expressed sequence tag (EST) and reverse transcription-polymerase chain reaction (RT-PCR) analyses suggested that two isoforms, a long and a short type, are translated from the *MAN* gene by alternative splicing (H. Takano, unpublished result). Interestingly, in the case of moss (*Physcomitrella patens*), two AN homologs (PpAN1 and PpAN2) were found by EST analysis

(M. Hasebe and H. Takano, unpublished data). While the deduced amino acid sequence of the PpAN1 gene contained the total region of plant AN protein, the PpAN2 protein excluded the C-terminus, and its size was similar to those of the short isoform of MAN and the drosophila dCtBP1 protein (H. Takano, unpublished data). If this holds true, the AN subfamily might play several roles in bryophytes. More detailed comparisons of homologs in the AN subfamily would also be informative, as some motifs found in AN are not conserved in moss homologs.

As a first step in such comparative studies, Cho et al[18] analyzed IAN, the AN homolog from *Ipomoea nil*. The genus *Ipomoea* belongs to the subclass Asterids, while *Arabidopsis* belongs to Rosids. IAN contains not only a D2-HDH motif, which is highly conserved within the CtBP family, but also LXCXE, NLS, and PEST motifs, which are specific to the AN subfamily. The expression of *IAN* cDNA driven by the cauliflower mosaic virus 35S promoter restored a defect in leaf expansion in the leaf-width direction in the *an-1* mutant of arabidopsis, as did the authentic *AN* cDNA, suggesting that IAN retains a common function with AN. By contrast, the complementation by IAN of a defect in the trichome branching pattern of the *an-1* mutant was less effective than that of the defect for leaf shape. These results suggest that the mechanisms by which *AN* regulates leaf width and trichome branching are separable, at least, in part.[18]

Perspective

Because of its enigmatic functions and behaviors, the CtBP/BARS family has been one of the hot topics in biology in recent years.[19,20] AN is the third subfamily of the CtBP/BARS complex found in the plant kingdom. Comparative and functional analyses of AN have just begun, but several unique features already have been discovered in the AN subfamily. Although AN is very similar to CtBP/BARS and it self-dimerizes, AN lacks the ability to associate with the PxDLS motif and possesses a unique long C-terminal domain. The mutation phenotype of the arabidopsis *an* mutant suggests that AN might be involved in cytoskeleton control.[6] Owing to its uniqueness, AN has interested researchers of authentic CtBP/BARS.[21] Considering both the unique features of AN and the commonalities between CtBP/BARS and AN, the AN subfamily might appropriately be treated as the third subfamily of the gene complex. In this vein, I propose to designate the whole gene family as the CBA family, or CtBP/BARS/AN family. Understanding of function(s) of AN subfamily is an important clue to reveal the fundamental role(s) of the CBA family in the organogenesis of multicellular organisms.

Recently, the *an* mutation phenotype in trichome branching was found to be rescued by the expression of drosophila *CtBP* driven by the cauliflower mosaic virus 35S promoter (S. Falk, M. Hülskamp, personal communication). This is an important clue to understand the common role of the CBA family. First, this data suggests that the AN-specific long C-terminal region might not be required for AN to function in trichome branching. Second, like arabidopsis, drosophila is an excellent system for analyzing gene function in organogenesis and individual development. Moreover, interacting partners of dCtBP are already well known in drosophila.[22,23] Therefore, comparative and functional analyses of AN with dCtBP, for example, by interchanging experiments between AN and dCtBP in transgenic arabidopsis and transgenic drosophila, could supply some important clues on the role and evolution of the enigmatic CBA family.

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