CIR, a corepressor linking the DNA binding factor CBF1 to the histone deacetylase complex

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ABSTRACT CBF1 is a member of the CSL family of DNA binding factors, which mediate either transcriptional repression or transcriptional activation. CSL proteins play a central role in Notch signaling and in Epstein–Barr virus-induced immortalization. Notch is a transmembrane protein involved in cell-fate decisions, and the cytoplasmic domain of Notch (NotchIC) targets CBF1. The Epstein–Barr virus-immortalizing protein EBNA2 activates both cellular and viral gene expression by targeting CBF1 and mimicking NotchIC. We have examined the mechanism of CBF1-mediated repression and show that CBF1 binds to a unique corepressor, CBF1 interacting corepressor (CIR). A CIR homolog is encoded by Caenorhabditis elegans, indicating that CIR is evolutionarily conserved. Two CBF1 mutants that were unable to bind CIR did not function as repressors, suggesting that targeting of CIR to CBF1 is an important component of repression. When expressed as a Gal4 fusion protein, CIR repressed reporter gene expression. CIR binds to histone deacetylase and to SAP30 and serves as a linker between CBF1 and the histone deacetylase complex.

Epstein–Barr virus (EBV) immortalizes B cells and is associated with human malignancies including Burkitt’s lymphoma, nasopharyngeal carcinoma, Hodgkin’s disease, and lymphoproliferative disease in immunosuppressed patients (1). Two major cell-signaling pathways are constitutively activated by EBV during immortalization. The EBV-latency membrane protein LMP-1 has effects on B cells similar to those that occur after activation of CD40, a membrane protein that is a member of the tumor necrosis factor receptor (TNFR) superfamily (2). LMP-1 functions as a constitutively activated TNFR. The cytoplasmic carboxyl terminus of LMP-1 binds to the TNFR-associated factors TRAF1, TRAF2, TRAF3, and TRADD, leading to activation of the transcription factor NF-κB (3–5). The EBV-encoded nuclear proteins EBNA2, EBNA3A, and EBNA3C each interact with the DNA binding factor, CBF1 (also called RBPJk and Jk) (6–11). EBNA2 activates both cellular and viral gene expression by interacting with a number of transcription factors through a combination of abolition of repression and the positive effects of an endogenous activation domain (17, 18, 24, 28). EBNA2 activates both cellular and viral gene expression by interacting with the CSL protein Su(H) and other CSL proteins. Notch signaling and in Epstein–Barr virus-induced immortalization and developmental pathways led to the isolation of a yeast two-hybrid screen to help identify the CSL protein Su(H) and other CSL proteins.

This paper was submitted directly (Track II) to the Proceedings office. Abbreviations: EBV, Epstein–Barr virus; CIR, CBF1-interacting corepressor; HDAC, histone deacetylase; TNFR, tumor necrosis factor receptor; EBNA, EBV-encoded nuclear antigen; IC, intracellular domain; hCIR, human CIR, TK, thymidine kinase; luc, luciferase; CMV, cytomegalovirus. Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF098297).

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RESULTS

Identification of CIR. The CBF1 ORF was fused to the Gal4 DNA-binding domain in pAS1CYH2, and a yeast two-hybrid screen for CBF1-interacting proteins was performed by using a B cell cDNA library cloned in pACT. Outgrowth on selective Trp and Leu medium followed by selection for yeast colonies expressing β-galactosidase led to the identification of two cDNA clones whose protein products showed strong interaction with CBF1. A database search revealed that both cDNAs encoded uncharacterized proteins. In subsequent experimental analyses, one of the proteins exhibited the properties of a corepressor. This novel 450-aa corepressor was designated CBF1 Interacting coRepressor (CIR).

In the database search, the predicted protein sequence of CIR isolated from the human B cell library matched the amino-terminal 135 amino acids encoded by a previously cloned and uncharacterized gene named recepin (gb U03644), but the predicted protein sequences diverged after amino acid 135. However, the cDNA sequences encoding the carboxyl terminus of hCIR completely matched other sequences in the expressed sequence database search (National Center for Biotechnology Information accession no. 1707048; ref. 70). Identical amino acids are indicated by the symbol = and conservative changes by (.). Spaces introduced to maximize alignment are shown as –. The amino acid numbers are provided on the right. (B) Northern blot analysis of hCIR expression performed by using a multiple-tissue RNA blot (CLONTECH) and a CIR DNA probe 32P-labeled by using random priming. After exposure to autoradiographic film, the blot was stripped and rehybridized with an actin DNA probe (actin). he, heart; br, brain; pl, placenta; lu, lung; li, liver; sm, skeletal muscle; ki, kidney; and pa, pancreas.

FIG. 1. (A) Alignment of the predicted protein sequences of hCIR and a C. elegans homolog (designated CeCIR) that was identified in a database search (National Center for Biotechnology Information accession no. 1707048; ref. 70). Identical amino acids are indicated by the symbol = and conservative changes by (.). Spaces introduced to maximize alignment are shown as –. The amino acid numbers are provided on the right. (B) Northern blot analysis of hCIR expression performed by using a multiple-tissue RNA blot (CLONTECH) and a CIR DNA probe 32P-labeled by using random priming. After exposure to autoradiographic film, the blot was stripped and rehybridized with an actin DNA probe (actin). he, heart; br, brain; pl, placenta; lu, lung; li, liver; sm, skeletal muscle; ki, kidney; and pa, pancreas.

screen for CBF1-interacting proteins was performed by using a B cell cDNA library cloned in pACT. Outgrowth on selective Trp −, Leu −, His − medium followed by selection for yeast colonies expressing β-galactosidase led to the identification of two cDNA clones whose protein products showed strong interaction with CBF1. A database search revealed that both cDNAs encoded uncharacterized proteins. In subsequent experimental analyses, one of the proteins exhibited the properties of a corepressor. This novel 450-aa corepressor was designated CBF1 Interacting coRepressor (CIR).

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tag database. Inspection of the recepin sequence revealed that the discrepancy was likely the result of inaccuracies in the recepin sequence that brought about a change of reading frame in the carboxyl-terminal portion of the predicted recepin protein. The 450-aa hCIR protein has a highly charged, serine-rich carboxyl terminus. The database search also revealed that a closely related protein is encoded by C. elegans (designated CeCIR; yk187f6.5). The 561-aa CeCIR shows striking sequence homology in the amino-terminal half of the protein, particularly over the first 150 amino acids. The carboxyl terminus, although diverged in primary sequence, retains the characteristic of being highly charged and serine-rich (Fig. 1A). Analysis of RNA expression indicated that, like CBF1, hCIR transcripts have a widespread tissue distribution, with strong expression in heart, skeletal muscle, and pancreas (Fig. 1B).

Intracellular Localization of CIR. CBF1 is a transcription factor that binds specifically to GTGGGAA motifs in responsive promoters. Complexes of factors involved in RNA synthesis and processing can, in some circumstances, be visualized as discrete intranuclear assemblies that appear as speckles or punctate spots in indirect immunofluorescence assays (reviewed in ref. 39). An example of such an assembly is the array of intranuclear speckles seen on staining for the SC35 splicing factor (40). We used indirect immunofluorescence to examine the intranuclear localization of CBF1 and CIR in transfected cells. Flag–CBF1 gave diffuse intranuclear staining with an underlying pattern of irregular strongly staining speckles plus strong staining in the nucleolus (Fig. 2 Left). Transfected CIR showed a similar pattern minus the nucleolar staining (Fig. 2 Center). Superimposition of the confocal images from cotransfected cells revealed coincidence between the majority of the CIR and CBF1 speckles (Fig. 2 Right). The immunofluorescence data show that CIR is a nuclear protein. The existence of speckles containing both CBF1 and CIR reinforces the suggestion from the yeast two-hybrid screen that these two proteins are capable of interaction and is consistent with both proteins contributing to a common transcriptional complex.

CIR Acts as a Repressor. CIR was next tested for transcriptional regulatory activity. Gal4 DNA-binding domain–CIR fusions were constructed in a mammalian expression vector. Cotransfection into HeLa cells of the CIR–Gal4 constructions with a CAT-reporter carrying upstream Gal4 binding sites revealed

Fig. 3. Mapping CIR domains required for repression and CBF1 interaction. (A) Transient-expression assay performed in a dose-response format in HeLa cells cotransfected with a 5xGal4BS–TKCAT reporter (5 μg), a TK-Luc control (1 μg), and increasing amounts of a CIR(1–450)–Gal4(1–95) expression vector. CIR represses reporter-gene expression in a dose-responsive manner. CAT, chloramphenicol acetyltransferase. (B) The repression domain is located within CIR amino acids 1–252. Transient-expression assay in HeLa cells cotransfected with 5xGal4BS–TKCAT reporter (5 μg), TK-Luc control (1 μg), and 2 μg of plasmids expressing the indicated CIR–Gal4(1–95) fusion constructions. The results shown are an average of three experiments, with the SD indicated. (C) The CBF1 interaction domain is also located within an N-terminal domain. Yeast two-hybrid assay using β-galactosidase induction as a measure of interaction. CIR(1–121), CIR(1–150), CIR(1–252), and EBNA2 (252–425) were expressed as Gal4ACT fusions, and CBF1 was expressed as a Gal4DBD fusion. The EBNA2–CBF1 pairing served as a positive control for a strong interaction. The results shown are an average of three experiments, with the SD indicated.
that CIR efficiently repressed reporter gene expression in a dose-dependent manner in this assay (Fig. 3A). A series of carboxyl-terminal deletions were introduced into CIR to locate the repression domain. When the resulting Gal4-fusion constructions were tested in the repression assay, the data indicated that wild-type levels of repression required CIR amino acids 1–252 (Fig. 3B). The fusion protein containing CIR amino acids 1–121 showed weak activity, whereas CIR(1–150) retained significant repression activity (8- to 10-fold). An examination of the requirements for CIR interaction with CBF1 by using the yeast interaction assay found that the CBF1 interaction domain was located within CIR(1–121) (Fig. 3C). The strongest signal was obtained with CIR(1–252) but CBF1 interaction was readily detectable with both CIR(1–150) and CIR(1–121) placing the CBF1 interaction domain also within the amino-terminal region of CIR. The amino-terminal 150 amino acids represent the region of highest sequence conservation between hCIR and CeCIR and the ability of CIR(1–150) to mediate repressive activity and CBF1 interaction implies that CeCIR is likely to function in a manner similar to hCIR.

**Tethering of CIR Is Necessary for CBF1-Mediated Repression.**

We had previously generated two CBF1 triple-alanine mutants altered at amino acids 233–235 from EEF to AAA and 249–251 from KLV to AAA [CBF1(EEF233AAA) and CBF1(KLV249AAA)] that showed loss of repression activity. The loss of repression did not reflect a global loss of functionality as each mutant retained interaction with EBNA2 (refs. 18 and 34; Fig. 4A). An equivalent mutation to CBF1(EEF233AAA) in Suppressor of Hairless, the *Drosophila* homolog of human CBF1, displays a hypomorphic hairless phenotype (41) correlating the activity measured in the transient expression assay with a developmental phenotype. The behavior of these two mutants implies that the region of CBF1 between amino acids 233 and 249 forms a key part of the CBF1 repression domain.

If CIR functions as a corepressor for CBF1, then the CBF1 EEF233AAA and KLV249AAA mutations would be expected to have an impact on CBF1–CIR interaction. The two CBF1 mutants and wild-type CBF1 were tested for interaction with CIR in the yeast interaction assay. The two CBF1 repression minus mutants showed a severely diminished ability to interact with CIR as indicated by a quantitative assay for β-galactosidase induction (Fig. 4B). The same loss of interaction was observed by using growth on selective His− medium as a measure of interaction as illustrated for the EEF233AAA mutant in Fig. 4C which also shows a qualitative β-galactosidase induction assay. These results correlate CIR interaction with the ability of CBF1 to function as a repressor.

**CIR Links an HDAC Complex to CBF1.**

Several mechanisms for transcriptional repression have been described, including DNA binding site competition, DNA bending, and destabilization of the TFIIID–promoter complex. An important recent advance in the understanding of transcriptional repression has been the realization that repressor proteins are frequently linked—either directly or indirectly through corepressors such as N-CoR, SMRT, and mSin3—to HDAC (reviewed in refs. 42 and 43). To address the mechanism of CBF1-mediated repression, a yeast two-hybrid assay was used to test for interaction with HDAC. The HDAC2–ACT construction used in this assay contained the same region of HDAC2 (amino acids 286–489) that was originally detected in a yeast two-hybrid screen as an interacting partner for YY1, a well characterized transcriptional repressor that interacts with the adenovirus E1A regulatory protein (38). CBF1 showed no interaction with HDAC2(286–489) (Fig. 5A) or with intact HDAC2 (data not shown). However, the combination of HDAC2–ACT and CIR–Gal4DBD led to induction of β-galactosidase synthesis, indicating interaction (Fig. 5A).

HDAC has been found previously to be brought into repression complexes through interactions with the corepressors N-CoR and mSin3. A subset of mSin3 complexes also includes SAP30 (44, 45). SAP30 was described only recently, and because CIR had not previously been recognized as a component of repression com-

![Fig. 4. CIR has the properties of a corepressor. (A) Representative transient-expression assay showing loss of repression by the CBF1 mutants CBF1(EEF233AAA) and CBF1(KLV249AAA). The assay was performed in HeLa cells by using a 5×Gal4HS-TKCAT reporter (5 μg), TK-Luc control (1 μg), and the indicated CBF1 constructions expressed as Gal4–DBD fusions. The relative specificity of the mutations is indicated by the continued ability of the mutant proteins to mediate EBNA2 activation. (B) CBF1(EEF233AAA) and CBF1(KLV249AAA) show diminished interaction with CIR in a yeast assay that uses quantitative β-galactosidase production as a measure of interaction. The wild-type CBF1 and the CBF1 mutants were expressed as fusions with the Gal4DBD and CIR(1–252) as a fusion with Gal4ACT. The results shown are an average of three experiments, with the SD indicated. (C) Illustration of the results obtained with CBF1(EEF233AAA) by using a qualitative assay for induction of β-galactosidase (Left) or growth on selective His− medium (Right) as a measure of interaction.](image)
experiments, with the SD indicated.

...EBNA2 (252–425) plus CBF1. The results shown are an average of three

...positive control for high-affinity interaction was

...negative control in

...interaction between CIR and HDAC2 (286–489) and SAP30 (41–221) expressed as a Gal4ACT fusions. The

...ratios plus HDAC, and the positive control for high-affinity interaction was EBNA2 (252–425) plus CBF1. The results shown are an average of three experiments, with the SD indicated.

DISCUSSION

The importance of histone deacetylation in mediating transcriptional repression has become increasingly clear with a recent example being the association between the retinoblastoma pro-

...tein and HDAC (46–48). The involvement of histone deacetylation in repression has been most extensively characterized for the Mad and nuclear hormone receptor family proteins where the corepressor complex includes SMRT or N-CoR, mSin3A or 3B, SAP30, SAP18, RbAp46 and RbAp46 in addition to HDAC1 and HDAC2 (49–54). In these examples, N-CoR or the related SMRT protein interacts with the DNA bound hormone receptor or MAD/MAX proteins. mSin3 in turn binds to N-CoR and brings in HDAC and the other mSin3 associated proteins SAP18, SAP30 and the histone binding proteins RbAp46 and RbAp48 (44, 45). SAP30 appears to function as a linker between N-CoR and mSin3. The amino terminus of SAP30 binds to N-CoR and the carboxyl terminus binds to mSin3 (45). Interestingly, SAP30 is present in only a subset of corepressor complexes. SAP30 is required for N-CoR-mediated repression through antagonist bound estrogen receptor and the POU domain protein PIF-1 but not for repression by the unliganded retinoic acid receptor or thyroid hormone receptor complexes (45). Here we describe a newly identified corepressor, CIR, that is a component of the CBFI-mediated repression complex and participates in the recruitment of HDAC to DNA-bound CBFI. CIR interacts strongly with SAP30 suggesting that SAP30 also participates in the CBFI associated repression complex. Because SAP30 is a component of the mSin3 complex, it seems likely that mSin3 will also be present in this complex although this was not addressed experimentally. The yeast two-hybrid experiments do not distinguish between direct and indirect contacts made through yeast homologs and hence it is also possible that there may be intermediate proteins between CBFI and CIR. Whether CIR is a general participant in all corepressor complexes containing SAP30 or is present in only a subset of complexes of which CBFI is representative remains to be investigated. CBFI-mediated repression is multifactorial. In addition to the recruitment of the HDAC complex, repression through destabilization of interactions between the transcription factors TFIID and TFIIA also has been recently described (55).

Overcoming CBFI-mediated repression is a crucial step in EBV immortalization of B cells and plays a role in the transmission of Notch signaling during development. The mechanism by which ligand-activated Notch signaling leads to transcriptional changes in the nucleus has been the subject of controversy. An understanding of the intracellular events that follow Notch signaling initially arose from the phenotypes observed in the presence of truncated Notch receptors. In particular, a truncated Notch composed only of the intracellular domain (NotchIC) translocates to the nucleus and functions as a ligand-activated receptor as judged by its gain-of-function phenotype (21). This activated form of Notch not only diverts cell-fate determination but also is associated with the development of tumors, including both naturally occurring and experimental T-cell neoplasms (30, 32).
56–59). It had been postulated that natural ligand-induced activation of Notch leads to a proteolytic cleavage event that releases NotchIC, but this proposal was not universally accepted. However, the recent identification of a proteolytic cleavage site between Notch1 amino acids G1743 and V1744 and the demonstration that cleavage at this site occurs in response to ligand-binding release of NotchIC along with evidence obtained by using a V1744 mutant that proteolytic cleavage at this site is necessary for complete activation of an HES-facilitator reporter (60), supports the model of Notch processing with nuclear translocation of NotchIC and interaction with CSL proteins in the nucleus.

Loss of CBF1 (mouse RBPF1; ref. 61) expression in mice is lethal to embryos at 10.5 days of gestation—an earlier stage than observed with Notch1 knockout mice—suggesting that CBF1 does not function solely as a downstream target of Notch but also plays a unique role in development (62). It is interesting to note that the CBF1 binding site, which ends in GGAGA, can overlap with an NFκB binding site, which starts with the sequence GGGA. Repression of genes whose promoters contain this sequence overlap could also be relieved by displacement of CBF1 for a consequence of the availability of increased levels of nuclear NFκB. Cellular promoters carrying CBF1 binding sites that overlap with binding sites for the transcription factor NFκB include interleukin 6, major histocompatibility complex class I, and interferon β (63–67). Notch signaling is evolutionarily conserved and has been demonstrated in C. elegans. The C. elegans Notch homologs are LIN-12 and GLP-1 and the CBF1 homolog is LAG-1 (14, 68, 69). The existence of a CIR homolog in C. elegans suggests that the pathways that utilize CIR as part of developmental gene regulation are highly conserved. The contribution of CIR apparently is not function solely as a downstream target of Notch but also plays a unique role in development (62). It is interesting to note that the CBF1 binding site, which ends in GGAGA, can overlap with an NFκB binding site, which starts with the sequence GGGA. Repression of genes whose promoters contain this sequence overlap could also be relieved by displacement of CBF1 for a consequence of the availability of increased levels of nuclear NFκB. Cellular promoters carrying CBF1 binding sites that overlap with binding sites for the transcription factor NFκB include interleukin 6, major histocompatibility complex class I, and interferon β (63–67). Notch signaling is evolutionarily conserved and has been demonstrated in C. elegans. The C. elegans Notch homologs are LIN-12 and GLP-1 and the CBF1 homolog is LAG-1 (14, 68, 69). The existence of a CIR homolog in C. elegans suggests that the pathways that utilize CIR as part of developmental gene regulation are highly conserved. The contribution of CIR apparently is not function solely as a downstream target of Notch but also plays a unique role in development (62).