We have previously shown that Ikaros can repress transcription through the recruitment of histone deacetylase complexes. Here we provide evidence that Ikaros can also repress transcription through its interactions with the co-repressor, C-terminal binding protein (CtBP). CtBP interacts with Ikaros isoforms through a PEDLS motif present at the N terminus of these proteins but not with homologues like Aiolos which lack this motif. Mutations in Ikaros that prevent CtBP interactions reduce its ability to repress transcription. CtBP interacts with Sin3A but not with the Mi-2 co-repressor and it represses transcription in a manner that is independent of histone deacetylase activity. These data strongly suggest that CtBP contributes to a histone deacetylase activity independent mechanism of repression by Ikaros. Finally, we show that the viral oncoprotein E1A, which binds to CtBP, also shows a strong association with Ikaros. This Ikaros-E1A interaction may underlie Ikaros's decreased ability to repress transcription in E1A transformed cells.

Gene targeting experiments in mice have firmly established that the zinc finger nuclear factors encoded by the Ikaros gene are essential for the development of the lymphoid arm of the vertebrate hemopoietic system (1). Mice homozygous for a null mutation in Ikaros lack B cells and their earliest described progenitors and are impaired in T cell differentiation (2, 3), whereas those homozygous for a dominant negative mutation in Ikaros lack all lymphocytes (4). The more severe phenotype of the dominant negative Ikaros mutation suggested the existence of homologues and led to the identification of Aiolos (5), Helios (6, 7), and Daedalus. In addition to its role in differentiation, Ikaros plays an important role in regulating proliferation. Mice expressing reduced levels of Ikaros exhibit augmented T cell receptor-mediated proliferative responses and develop leukemias and lymphomas with complete penetrance (8, 9). Thus, Ikaros is an essential regulator of lymphocyte differentiation and homeostasis. Aiolos plays a similar role to Ikaros in the B cell lineage (10).

The Ikaros gene encodes by means of alternative splicing at least eight different isoforms all of which contain a bipartite activation domain (11–13) and two C-terminal zinc fingers that are involved in homo- and heteromeric interactions (12). Ikaros isoforms differ in the number of N-terminal DNA-binding zinc fingers that consequently differentiate them into DNA binding and non-DNA binding proteins (11, 13). We and others have shown that the DNA-binding isoforms of Ikaros can activate transcription of genes when bound in cis to Ikaros-binding sites (11, 12, 14). In contrast, Ikaros represses transcription when recruited to DNA through a heterologous DNA-binding domain (15). This repression is cell type- and promoter-specific and is effected through two repression domains which interact with the mSin3A (15) and Mi-2 proteins (16) which are components of two distinct histone deacetylase complexes (17–20). Consistent with a role for histone deacetylases in Ikaros-mediated repression, histones are underacylated in the vicinity of Ikaros recruitment sites and the histone deacetylase inhibitor, trichostatin, alleviates repression by Ga4-Ikaros (15).

In addition to histone deacetylase-dependent mechanisms of repression used by transcription factors such as MAD (21), the unliganded nuclear receptor proteins (22) and MTR (23), histone deacetylase-independent mechanisms of repression have been reported for Xenopus Polycomb (24). Recent studies also indicate that some repressors like Rb2 (25), mSin3A (26, 27), c-Myc promoter binding protein (28), and RBP1 (29) may utilize both of these strategies of repression. Another co-repressor that may fall into this category is the C-terminal-binding protein, CtBP, so named for its identification as an interactor of the C-terminal of adenovirus E1A (30, 31). In vivo studies have established that deletion of the region of E1A that facilitates its interaction with CtBP significantly increases the transforming and tumorigenic potential of this oncoprotein (30).

At least two highly related CtBP proteins, CtBP1 and CtBP2, have been identified in vertebrates (32, 33). CtBP1 transcripts are expressed in most human tissues at approximately the same level as CtBP2 transcripts except in the thymus and peripheral blood leukocytes where the latter are hardly detectable (34). The mechanism by which CtBP effects repression is largely unclear. Based on the observation that several Dro sophila repressors that recruit CtBP work over distances of less than 100 base pairs to inhibit the core promoter, it has been suggested that CtBP may function as a short range repressor (35). There is evidence from overexpression studies that CtBP can interact with histone deacetylase 1 (36) and that in some cases repression by Ga4-CtBP can be relieved by deacetylase

* This work was supported in part by National Institutes of Health Grant RO1 AI33062 (to K. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a Howard Hughes Medical Institute predoctoral fellowship. Present address: Graduate program in Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138.

¶ Stohlman Scholar of the Leukemia Society of America. To whom correspondence should be addressed: CBRC/IGH, East, Bldg. 149, 13th St., Charlestown, MA 02129. Tel.: 617-726-4445; Fax: 617-726-4453; E-mail: katia.georgopoulo@cbrc2.mgh.harvard.edu.

1 T. Ikeda and B. Morgan, personal communication.

2 The abbreviations used are: Rb, retinoblastoma; CtBP, C-terminal-binding protein; HDAC/HD, histone deacetylase; Ik, Ikaros; RBP1, Rb-binding protein 1; CIP, CtBP interacting protein; tk, thymidine kinase; AdMLP, adenovirus major late promoter; GST, glutathione S-transferase; HA, hemagglutinin; MT, Myc epitope tag; CAT, chloramphenicol acetyltransferase; GH, growth hormone.
HDAC Activity-independent Mechanism of Repression by Ikaros

Inhibitors (37). CtBP has also been described to repress promoters that are unresponsive to trichostatin (38).

CtBP is targeted to DNA through its association with transcription factors containing a sequence that is similar to the consensus motif, PXDLS (31, 39). A number of transcription factors with this motif have been shown to interact with CtBP. These include transcription factors such as Knirps (35) and Hairy (40) in Drosophila and basic Kruppel-like factor (32), Xenopus T cell factor 3 (41), Xenopus Polycomb 2 (34), Net (37), FOG (42), and CtIP (39, 43) in vertebrates. Mutations in the PXDLS motif that prevent interaction with CtBP have been shown to decrease the ability of several of these proteins to repress transcription both in vivo and in vitro (32, 34, 35, 40). Genetic studies in flies have shown that CtBP is required for appropriate segmentation and dorso-ventral patterning (35, 40) while similar studies in Xenopus also provide a role for CtBP later in the development of head and notochord structures (41).

In this report, we provide evidence that Ikaros can repress transcription through its interaction with CtBP. This is the first functional difference between Ikaros and its family members which cannot interact directly with CtBP. Although CtBP can interact with Sin3A/HDAC but not with Mi-2/HDAC, CtBP continues to repress transcription even in the presence of histone deacetylase inhibitors. Finally, we show that Ikaros association with the adenosine oncprotein E1A alleviates its ability to repress transcription.

EXPERIMENTAL PROCEDURES

Plasmids—Flag-Ik1, Flag-Ik1cm, Flag-hCtBP1, Flag-CtN1, -CtN2, -Cc1, -Cc2, and -Ct3 were constructed by standard cloning techniques in the pCMV2-Flag vector (Sigma). Mutations in the CtBP interaction domains of Ikaros were generated by a polymerase chain reaction based approach. BXG1-hCtBP1, the related N- and C-terminal deletions of hCtBP1 and different domains of Ikaros lacking or containing mutations in the CtBP interaction domain were constructed by cloning the relevant regions into the BXG1 vector (15) which encodes the Gal4 DNA-binding domain (amino acids 1–147). CD8M-Ik1, -Ik2, -Ik3, -cc1, and -Ct3 were expressed as recombinant proteins in E. coli.

Transfections—293T, NIH3T3, and U2OS cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (Hyclone). Transfections of these cell lines were carried out using the HBS-CaPO4 method. For activation assays, 0.25 μg of Flag-tagged Ikaros plasmids, 3 μg of the 4XIKBS2tkCAT reporter, and 0.5 μg of the pXGH5 hormone transcription efficiency control plasmid were used. For repression assays, 0.5 or 1 μg of the Gal4-fusion plasmid, 5 or 10 μg of the Gal4-reporter plasmid, and 0.5 μg of the pXGH5 control plasmid were used. Twenty-four hours after transfection, cells were fed with fresh medium and 18–24 h later cells were harvested and processed for CAT assays as described (15). In those instances where trichostatin A (Upstate Biotech) was employed, we added the drug to the cells 16–18 h before harvesting. GH assays were done as recommended by the manufacturer (Nichols Institute). Transfections were typically performed in duplicate and repeated between three and six times.

Immunoprecipitation and Western Analysis—Whole cell extracts from 293T cells transfected with the relevant plasmids were prepared as described previously (15) and pre-cleared using Protein G-agarose beads (Roche Molecular Biochemicals). The pre-cleared extracts were incubated with the antibody of interest or the relevant isotype control on ice for 1 h. 30 μl of Protein G beads were then added to the extract and the extracts were rotated overnight. The beads were collected by centrifugation and washed four times with TS buffer (12). The beads obtained after this procedure were treated with SDS sample buffer, boiled at 95 °C for 15 min, and loaded on a SDS-polyacrylamide gel along with 8–10% of the cell extract used for the immunoprecipitation. The proteins were transferred to a nitrocellulose membrane, probed with the relevant antibody, and examined by autoradiography with ECL (Amersham Pharmacia Biotech). Antibodies used were: T7 (Novagen), Myc tag (Roche Molecular Biochemicals), HA (Ab2Co), Flag M2 (Sigma), Gal4, Sin3B (Santa Cruz), HDAC2 (Zymed Laboratories Inc.), E1A (OncoGene Science), and anti-Ikaros, Aiolos, and Mi-2 which have been previously described (5, 12, 16).

GST Interaction Assays—GST and GST-hCtBP1 were prepared as described previously (39). Preparation of thymic nuclear extracts was essentially as described in Ref. 11. 1–2 μg of the GST proteins were incubated with thymic nuclear extracts or reticulocyte lysate (Promega) for 1 h at 4 °C and washed extensively with MT-phosphate-buffered saline (PBS). The GST beads were then washed in SDS sample buffer and fractionated on an SDS-polyacrylamide gel. Immunoblotting was done as described in the previous section.

Histone Deacetylase Assays—Histone deacetylase assays were performed on tritiated chicken reticulocyte histones as described (44). Briefly, immunoprecipitates from 293T whole cell extracts were washed 3× in TS buffer and incubated with 100,000 cpm of tritiated acetylated histones for 45 min at 30 °C in HD assay buffer. The reaction was stopped by acidification and the released tritium was extracted with ethyl acetate.

RESULTS

Ikaros, but Not Its Family Members, Interacts with CtBP—We have previously reported that Ikaros and Aiolos, when fused to the GAL4 DNA-binding domain, can function as transcriptional repressors in a promoter and cell type specific manner. The repression mediated by Gal4-Ikaros and Gal4-Aiolos on the adenovirus major late promoter (AdMLP) was found to be significantly relieved but not abolished by the histone deacetylase inhibitor, trichostatin A. Thus, we argued that histone deacetylase recruitment serves as one mechanism for Gal4-Ikaros and Gal4-Aiolos repression on the AdMLP. In agreement with these findings, Ikaros was found to associate with the NURD and Sin3 histone deacetylase complexes.

A careful analysis of the primary sequence of the murine Ikaros protein led to the identification of a 5-amino acid sequence, PEDLS, as an amino terminus (Fig. 1A), that matches the consensus recognition motif for the C-terminal-binding protein co-repressor, CtBP (30, 31). This sequence is conserved between all the described Ikaros orthologs found in chicken, rainbow trout, and zebra fish but is not present in the Ikaros family members: Aiolos, Helios, and Daedalus (Fig. 1A). However, both Daedalus and Helios contain the sequences, PELDG and PISLI, respectively, in their last exon, that varies significantly from the well established consensus CtBP interaction motif.

Given the putative CtBP-binding site in Ikaros, we tested their interaction using bacterially purified GST-hCtBP1 and thymic nuclear extracts and reticulocyte lysates. We have restricted our study to hCtBP1 since CtBP2 is not expressed in the thymus (34). GST-CtBP but not the GST fusion partner interacted with endogenous Ikaros protein from both sources (Fig. 1B). Importantly, a small amount of CtBP2 was also found to be present in Ikaros complexes purified from T cell nuclear extracts (Fig. 1C) (16). Thus, Ikaros can interact with CtBP both in vitro and in vivo.

To examine the importance of the PEDLS motif in Ikaros-CtBP interactions, we constructed two mutations. In the first, we deleted this 5-amino acid region (Ik1d(PEDLS)) and in the second we mutated the core amino acids DL → AS (Ik1cm) in the context of the full-length protein. We transfected these mutants with hCtBP1 tagged with T7 into 293T cells and performed immunoprecipitations. Neither of the Ikaros mutants was able to interact with CtBP (Fig. 2A). We then tested the impact of these mutations on the ability of Ikaros to associate with components of the NURD and Sin3 histone deacetylase complexes. Both of the Ikaros mutants interacted with Mi-2g, Sin3A, Sin3B, and HDAC2 in a fashion indistinguishable from wild type (Fig. 2B and data not shown for Sin3B). Consistent with these results, Flag-Ikaros or Flag-Ik1cm
munoprecipitated from transfected 293T cells had similar histone deacetylase activities (Fig. 2E). These data underscore the importance and specificity of the PEDLS motif in Ikaros-CtBP interactions. In addition, it shows that the ability of Ikaros to interact with CtBP or its lack thereof does not influence its interactions with histone deacetylase complexes.

At least eight different splice variants of Ikaros have been described (11–13). Using the 293T transfection/immunoprecipitation approach we tested whether hCtBP1 could distinguish between these proteins. All tested isoforms including the non-DNA binding isoform, Ik7, interacted with CtBP (Fig. 2C). This was expected since all isoforms contain the PEDLS motif. In addition, point mutations of the C-terminal zinc fingers of Ikaros that abolish formation of a higher order complex (12) had no effect on Ikaros interactions with CtBP (data not shown).

We next examined whether the Ikaros family members, Aiolos, Helios, and Daedalus could interact with CtBP by co-expressing these proteins in 293T cells. None of these proteins interacted with CtBP and Ikaros in primary activated T cells. Immunopurification of Ikaros-containing complexes was accomplished using a FlagM2 column. The unbound proteins (S), final wash (W3), and eluate (E) were tested by immunoblot analysis (IB) with antibodies to CtBP.

FIG. 1. Ikaros can interact with CtBP. A, a diagrammatic representation of the Ikaros protein and the location (*) of a sequence with similarity to the consensus CtBP interaction motif. Exons 1/2, 3, 4, 5, 6, and 7 are indicated as horizontal rectangles and the zinc fingers are represented as vertical rectangles. A comparison of the sequences found at the N-terminal of Ikaros and its homologues is provided. Capitalized letters indicate a match to the consensus CtBP motif. B, in vitro interaction between Ikaros and hCtBP1. GST interaction assays between CtBP and Ikaros from thymic nuclear extracts and reticulocyte lysates as determined by immunoblot (IB) analysis. I, input (5% for thymic extract and 25% for reticulocyte lysate); G, GST, GCtBP, GST-hCtBP1. Molecular weight markers are provided in kDa at the left. C, in vivo interaction between CtBP and Ikaros in primary activated T cells. Immunopurification of Ikaros-containing complexes was accomplished using a FlagM2 column. The unbound proteins (S), final wash (W3), and eluate (E) were tested by immunoblot analysis (IB) with antibodies to CtBP.

FIG. 2. Ikaros interactions with hCtBP1 require an intact PXDLS motif. A, 293T cells were co-transfected with expression vectors for T7-HcCtBP1 (10 μg) and wild type or CtBP interaction site mutant (Ik1cm) Ikaros (10 μg of untagged plasmid or 1 μg for Flag-tagged plasmid). Immunoprecipitations (IP) were performed on whole cell lysates with T7 antibodies. Immunoblot analysis (IB) with Ikaros revealed differences in interactions between wild type and mutant Ikaros proteins with hCtBP1. Blots were stripped and reprobed with the Ab used for immunoprecipitation to ascertain that the epitope-tagged proteins were immunoprecipitated (control). I, input; C, isotype control IP; B, bound fraction from specific IP. B, the interaction of Mi-2, MT-Sin3A, and endogenous HDAC2 with wild type and mutant Ikaros proteins were tested as in A. IPs were performed using Flag antibody. C, interaction between T7-hCtBP1 and Ikaros isoforms were tested as in A. D, interaction between T7-hCtBP1 and Ikaros family members, Aiolos, Daedalus, and Helios were tested as in A. E, histone deacetylase assays of Flag immunoprecipitates obtained from 293T extracts transfected with Flag-Ik1 and Flag-Ik1cm. Assays were done twice in duplicate with or without trichostatin A.
section we provided evidence that Ikaros but not Aiolos can interact with CtBP. Thus, we were interested in determining whether CtBP could exist in association with Ikaros and Aiolos or whether it was excluded from their complex. The three proteins were co-expressed in 293T cells and immunoprecipitated using the Flag epitope attached to CtBP. As a control for this analysis, we transfected Ik1cm (an Ikaros-CtBP interaction mutant) with Aiolos and CtBP. Immunoprecipitation of cell extracts containing wild type Ikaros but not of extracts containing the mutant variant, Ik1cm, brought down Aiolos (Fig. 3A). Thus Ikaros, Aiolos, and CtBP can exist in a ternary complex when expressed in the same cell. Using the same procedure, we next tested whether Mi-2β, a component of the NURD histone deacetylase complex could exist in a ternary complex with Ikaros and CtBP. Unlike Aiolos, no interactions were seen between the Ikaros-CtBP complexes and Mi-2β (Fig. 3B). Immunopurification of Flag-CtBP complexes from 293T cells revealed that CtBP associates with a small amount of endogenous Sin3A and HDAC2 regardless of whether wild type Ikaros or Ik1cm was present (Fig. 3C). These findings were independently confirmed by GST interaction assays (data not shown).

In summary, Ikaros and CtBP can exist in a ternary complex with Aiolos but not with Mi-2β, a component of the NURD histone deacetylase complex. Also, CtBP co-purifies with a small fraction of endogenous Sin3A and HDAC2 in the absence of Ikaros.

**hCtBP1 Interacts with Histone Deacetylases**—The Ikaros-independent ability of CtBP to interact with histone deacetylases was further investigated and was found to be heavily influenced by epitopes attached to its N terminus; T7 tagged CtBP does not interact with HDAC2 and Flag-CtBP interacts weakly whereas the strongest interaction was seen when CtBP was fused to the Gal4 DNA-binding domain (data not shown). Consistent with the CtBP interactions with HDAC2, Gal4-CtBP was found to have significant histone deacetylase activity (Fig. 3E).

CtBP interactions with endogenous HDAC2 were tested in thymic nuclear extracts and rabbit reticulocyte lysates. Although HDAC2 was present in both extracts at roughly similar levels, only HDAC2 derived from the thymic extracts interacted with GST-CtBP (Fig. 3D). Possible interactions with other factors or differences in post-translational modifications in these two cell types may account for these results.

Thus, CtBP can interact with HDAC2 and this interaction may be dependent on the cell type. In addition, structural perturbations at the N terminus of CtBP may alter its ability to interact with HDACs and other factors. Furthermore, interactions of CtBP with HDAC2 and Sin3A are possibly indirect since both proteins lack a PXDLS motif.

**Delineation of Domains on hCtBP1 That Interact with Ikaros and HDAC2**—In order to identify the minimal region(s) of hCtBP1 required for its interactions with Ikaros and HDAC2, we generated two series of N- and C-terminal deletions of hCtBP1, one of which was Flag tagged while the other was GST tagged to Flag, HDAC2, and Sin3A. These data suggest that the first 95 amino acids of CtBP1, although insufficient for binding,
can contribute to binding Ikaros along with amino acids 179 through 196. These data suggest a complex mode of interaction between CtBP and Ikaros that involves more than one domain on CtBP1 and which may be influenced by protein folding and(or) other interactions.

Gal4-CtBP interactions with endogenous HDAC2 present a somewhat similar picture (Fig. 4, A and C). HDAC2 interacts most strongly with full-length hCtBP1 and only barely above background levels with either the N- or C-terminal deletion series of CtBP1 (Fig. 4C). Thus, more than one domain on hCtBP1 is required for its interaction with Ikaros and HDAC2.

Gal4-hCtBP1 Represses Transcription Independent of Histone Deacetylase Activity—We have found that Gal4-hCtBP1 can repress transcription of the tk and AdML promoters in several cell lines (Fig. 5, B and C, and data not shown). Since Gal4-hCtBP1 represses transcription in cell lines in which Ikaros is not expressed its mode of repression is Ikaros-independent. This suggests that as long as CtBP can be recruited to a target promoter through Ikaros, Gal4, or another DNA bound transcription factor, it can effectively repress transcription. The ability of Gal4-hCtBP1 to repress transcription is decreased when it is recruited to Gal4 sites at increasing distances from the tk promoter (data not shown). This is in agreement with studies on Drosophila CtBP which implicate it in short range repression events (45).

To delineate the region on hCtBP1 required for its transcriptional repression properties, we transfected 293T cells with a Gal4tkCAT reporter and the Gal4-hCtBP1 deletion series described in the preceding section. Only the full-length protein and Gal4-CtN1 repressed transcription of the tk promoter (Fig. 5A). However, Gal4-CtN1 interactions with HDAC2 and Ikaros are barely detectable (Fig. 4, B and C), suggesting that repression by CtBP fusion’s in this assay is independent of histone deacetylase activity or interactions with Ikaros. Surprisingly, the Gal4-CtN3 deletion mutant activated transcription by 2-fold from this promoter.

To further explore whether the mechanism of CtBP repression relies on histone deacetylase activity, we tested repression of the tk and the AdML promoters in NIH3T3 cells in the presence and absence of the histone deacetylase inhibitor, trichostatin A. Repression by Gal4-hCtBP1 on both the tk and AdMLP was independent of histone deacetylase activity in contrast with the repression effected by Gal4-Sin3BSF used as a control (Fig. 5B). These findings are in agreement with our previous observation that a deletion mutant of CtBP1, CtN1, which barely interacts with HDAC, maintains its ability to repress transcription (Fig. 4C and 5A). We have varied the amount of effector to reporter as well as performed transfections in other cell types but have not observed any significant CtBP de-repression in the presence of trichostatin (data not shown).

Thus, although hCtBP1 can interact with HDACs, it is capable of repression using mechanisms independent of the enzygmatic activity of histone deacetylases. Its ability to repress is also independent of Ikaros suggesting that the repression is effected directly by CtBP1 possibly through its interactions with other corepressors and/or the RNA-polymerase complex. This supposition is strengthened by our recent findings that
CtBP1 can interact with at least two components of the basal transcriptional machinery (data not shown).

**Mutations in Ikaros That Prevent Interactions with CtBP**

To investigate the functional consequence of Ikaros interactions with hCtBP1, we made Gal4 fusions of the full-length and subregions of Ikaros that contain the wild type PEDLS motif or the mutant variant PEasS (suffix "cm") that cannot interact with hCtBP1 (Fig. 2A). These plasmids were transfected into 293T cells and extracts prepared from these cells were tested with antibodies to Gal4 to ensure that the proteins encoded by these vectors were made at similar amounts (Fig. 6D). They were then individually transfected along with either the G5tkCAT or G5AdMLPCAT reporter into U2OS and NIH3T3 cells and their effect on transcription was assayed.

A mutation of the CtBP-binding site on Ikaros, that prevents its interaction with the co-repressor, significantly reduced its ability to repress transcription on both the tk and AdML promoters (Fig. 6, A and B, compare BXG1-Ik1 to BXG1-Ik1cm). The effects of the CtBP-binding site mutation on repression were also observed in the context of the subregions of Ikaros (Fig. 6, A and B, compare BXG1-Ik1 to BXG1-Ik1cm and BXG1-N1 to BXG1-N1cm). These effects on repression were similar in both U2OS and NIH3T3 cells (data not shown). We take these data to mean that Ikaros mutants that cannot interact with CtBP are incapable of recruiting a putative CtBP corepressor complex to a target promoter to effect repression. It is important to emphasize here that the Ikaros mutation that prevents interactions with CtBP alleviates but does not abrogate Ikaros’s repression capabilities. This should not be surprising since Ikaros containing the CtBP interaction site mutation can still associate with the Sin3 and NURD histone deacetylase complexes (Fig. 2B).

We next compared untagged Ikaros wild type and CtBP interaction mutant proteins for their ability to activate transcription from Ikaros-binding sites. NIH3T3 cells were transfected with wild type or mutant Ikaros plasmids and a reporter containing four Ikaros-binding sites (4XIkBS2 tkCAT). No significant difference was observed between the wild type or the mutant proteins in their ability to activate transcription from their own sites remains largely unaffected by these mutations.

**Ikaros Interactions with Adenovirus E1A Reduces Its Ability to Repress Transcription**

CtBP was first identified as a protein that interacted with Exon 2 of adenovirus E1A (30, 31, 46). Since CtBP interacts with Ikaros, we were interested in determining whether E1A could associate with Ikaros through CtBP. Extracts from 293T cells transfected with Flag-hCtBP1 (as a positive control), Flag-Ik1, and Flag-Aio3 were immunoprecipitated with Flag and E1A antibodies. Unexpectedly, Flag-Ikaros and -Aiolos proteins brought down more E1A proteins relative to Flag-Ik1cm and Flag-Aio3 were immunoprecipitated with Flag and E1A antibodies. To next test the ability of Ik1cm, which is incapable of interacting with CtBP1, to associate with E1A proteins, Ik1cm and Ik1 immunoprecipitated roughly equal levels of E1A indicating that this protein association was not brought about through CtBP (Fig. 7B).

The functional relevance of the Ikaros-E1A interaction was examined in transient expression studies. U2OS cells (which lack any viral oncoprotein) were transfected with Gal4 or Gal4-Ik1 and G5tkCAT and increasing amounts of 13SE1A and

---

**Fig. 5. Histone deacetylase activity is not required for repression by hCtBP1.** A. 293T cells were transfected with BXG1 or BXG1-hCtBP1 subregions (1 μg), the reporter, G5tkCAT (10 μg), and a GH plasmid (0.05 μg) to control for the transfection efficiency. CAT activity was corrected for transfection efficiency using the GH assay. Fold Repression was calculated by dividing the decrease in CAT activity (measured in counts/min) of the BXG1-hCtBP1 subregions by the CAT activity (measured in cpm) of BXG1. B. NIH3T3 cells were transfected with 10 μg of either G5tkCAT (left panel) or G5AdMLPCAT (right panel), 1 μg of the indicated Gal4 effector plasmids and 0.5 μg of a growth hormone plasmid to correct for transfection efficiency. Sin3BSF is a short form of Sin3B that interacts with histone deacetylase. Transfectants were left untreated or treated with trichostatin A (100 ng/ml) 16–18 h before harvest. Corrected CAT activity was calculated as in A. Fold derepression upon trichostatin A treatment is indicated below the graph and was calculated as the increase in normalized CAT activity upon trichostatin treatment divided by the corrected CAT activity in untreated cells.
assayed for their effect on Ikaros transcriptional repression. In the presence of E1A, repression by Gal4-Iκ1 was relieved by 7-fold (Fig. 7C, inset). A much smaller (2-fold) effect on the transcriptional activity of the Gal4 DNA-binding domain was seen in the presence of E1A (Fig. 7C, inset). These findings are consistent with the reduced repression capabilities of Ikaros proteins in the E1A transformed cell lines, 293 and 293T (15). Thus, viral oncoproteins may target Ikaros and its family members and in the process disrupt their normal interactions and alter their ability to repress transcription.

**DISCUSSION**

In this study we provide evidence for an interaction between the hemolymphoid zinc finger transcription factor Ikaros and the co-repressor CtBP. We demonstrate that although CtBP is capable of interactions with histone deacetylases, it mediates
repression even in the presence of the deacetylase inhibitor, trichostatin. These findings argue for a histone deacetylase activity-independent mode of repression by Ikaros proteins. Taken together with our previous findings (15, 16), these data provide compelling evidence that Ikaros can act as a repressor of transcription by utilizing both histone deacetylase activity-dependent and -independent mechanisms.

Utilizing distinct repression mechanisms that include direct interference with holoenzyme recruitment and chromatin compaction events may be necessary for effective down-regulation of gene expression. Since the description of Rb as a repressor that uses both HDAC-dependent and -independent means of repression (25), several transcription factors including SMRT (26), N-CoR (27), Net (37), and RBP1 (29) have been included in this category. In addition to their deacetylase-dependent modes of repression, SMRT and N-CoR interact with TFIIB and presumably affect the formation of a functional preinitiation complex (26, 27) while RBP1 and Net have two repression domains one of which recruits deacetylases while the other utilizes alternative repression mechanisms (29, 37).

Here we have shown that Ikaros and its orthologs contain a PEDLS motif and that CtBP interacts with Ikaros proteins through this sequence. Interestingly, none of the Ikaros family members contain this motif or interact with CtBP yet they can still exist in a ternary complex with Ikaros and CtBP. In contrast to the 5 amino acids on Ikaros required for interaction with CtBP, a larger region within the N-terminal 300 amino acids of CtBP is required for its association with Ikaros.

CtBP was originally identified as an interactor of the adeno-viral oncoprotein, E1A, and was found to reduce the oncoproteins ability to transform cells (30). In testing whether Ikaros proteins could participate in an E1A-CtBP complex, we found that Ikaros-E1A interactions occur independently of CtBP. Viral oncoproteins facilitate cell transformation through their interaction with regulatory factors and by interfering with their normal functions (47–49). Ikaros interactions with E1A were found to significantly alter its ability to repress transcription. Interestingly, the Ikaros repression potential is greatly reduced in various virally transformed cell lines such as 293T (transformed by E1A and SV40 T Ag), COS (transformed by SV40 Tag), and HeLa (transformed by HPV E7). It is likely that Ikaros may also serve as a target for several viruses that target lymphoid cells and cause their transformation by interfering with Ikaros activity.

We have previously shown that Ikaros can interact with the Sin3 and NURD histone deacetylase complexes (15, 16). Mutations in Ikaros that prevent interactions with CtBP do not affect Ikaros’s ability to associate with Sin3, Mi-2, or HDAC2. These data suggest that CtBP is not required for Ikaros association with these histone deacetylase complexes and implicates a different mechanism of repression.

How does CtBP effect repression? CtBP was originally recognized to have significant homology with the NAD-dependent D-isomer specific 2-hydroxy acid dehydrogenases which suggested a role for this enzymatic function in repression (31). However, thus far CtBP has not been demonstrated to have any significant dehydrogenase activity (31) and a mutation in the active site of the dehydrogenase domain does not affect transcriptional repression (32). At least two studies have suggested a histone deacetylase dependent mode of repression: 1) co-immunoprecipitation of CtBP and HDAC1 from U2OS cells and in vitro binding experiments have shown that CtBP can interact with HDAC1 (36, 2) trichostatin was found to relieve Gal4-CtBP repression in CHO cells (37). In this report, we also show that CtBP can interact with endogenous HDAC2 from 293T cells and thymic nuclear extracts. In addition, we find that CtBP can interact with endogenous Sin3A but not with Mi-2, which are components of two distinct HDAC complexes. However, we show that despite the observed interactions between CtBP, Sin3, and HDAC2, the repression capabilities of CtBP are not dependent on HDAC enzymatic activity. Repression of two promoters by Gal4-CtBP was not alleviated by the deacetylase inhibitor, trichostatin, in 293T or in NIH 3T3 cells. In addition, the minimal repression domain on CtBP did not interact with HDAC2 at any significant level. In agreement with our findings of a histone deacetylase activity-independent mode of repression by CtBP, a recent report on Rb repression mechanisms has implicated the CtIP-CtBP complex in the Rb HDAC Rb’s HDAC-independent repression function (38). In summary, our data suggest that the C-terminal-binding protein can repress transcription through mechanisms other than the recruitment of histone deacetylase activity. However, our data does not rule out the possibility that CtBP may repress transcription through deacetylases that are refractory to the inhibitor trichostatin and/or through interactions of histone deacetylases with other co-repressors or the basal transcriptional machinery.

Ikaros appears to repress transcription through its interaction with histone deacetylases and through means other than the recruitment of deacetylase activity. Ikaros represses the MLP promoter largely through the action of histone deacetylases (15). However, Ikaros repression of the AdML promoter is not completely relieved by trichostatin treatment suggesting an additional role for histone deacetylase-independent mechanisms of repression on this promoter. In addition, Ikaros-mediated repression of the tk promoter is independent of histone deacetylase activity. MUTATION OF THE CtBP MOTIF ON Ikaros significantly reduced Ikaros’s ability to repress both the tk and MLP promoters. Taken together with the HDAC independent repression by CtBP on these two promoters, we conclude that CtBP is one component of the histone deacetylase-independent mechanism of repression utilized by Ikaros. Ikaros can also interact with mSin3 proteins (15). The ability of mSin3A to repress transcription was largely unaffected by the deletion of its histone deacetylase interaction domain suggesting that it can also repress through histone deacetylase-independent mechanisms (21). Further support for this suggestion and the underlying mechanism has been recently provided (26, 27). Thus, both CtBP and Sin3 interactions with Ikaros may play a role in Ikaros’s histone deacetylase activity-independent mechanisms of repression.

What are the possible in vivo consequences of Ikaros interactions with CtBP? Ikaros is the earliest expressed member of its family during hemopoiesis (1). The Ikaros-CtBP association may play an important role in regulating hemopoietic lineage commitment decisions. Lineage commitment requires the activation of lineage specific genes as well as the down-regulation of non-lineage genes which are expressed in the early progenitor cells. Ikaros-CtBP interactions could help rene and restrict committed cells to the lymphoid lineage. Genetic experiments in flies have suggested a role for CtBP in early in development (40) while similar studies in Xenopus have identified additional roles for this protein later in development (41). CtBP has been shown to interact with Xenopus Polycomb 2 and a small fraction of CtBP in U2OS cells was also found to co-localize with Polycomb nuclear domains (34). Polycomb proteins are components of an elaborate machinery that serves to heritably maintain the silenced state of genes (50). Thus Ikaros-CtBP interactions may serve to recruit Polycomb group proteins to maintain the silenced state of non-lineage and de-
developmentally regulated genes. A conclusive description of the role of Ikaros-CtBP interactions in lymphocyte development will need to await future studies which will include generation of “knock-in” mice carrying Ikaros mutant alleles that cannot interact with CtBP.

Acknowledgments—We thank our colleagues who have generously provided us with reagents.

REFERENCES
1. Cortes, M., Wong, E., Koipally, J., and Georgopoulos, K. (1999) Curr. Opin. Immunol. 11, 167–171
2. Wang, J., Nichogiannopoulou, A., Wu, L., Sun, L., Sharpe, A., Bigby, M., and Georgopoulos, K. (1996) Immunity 5, 537–549
3. Winandy, S., Wu, L., Wang, J. H., and Georgopoulos, K. (1999) J. Exp. Med. 190, 1039–1049
4. Georgopoulos, K., Bigby, M., Wang, J. H., Melnár, Á., Wu, P., Winandy, S., and Sharpe, A. (1994) Cell 79, 143–156
5. Morgan, B., Sun, L., Avitahl, N., Andrikopoulos, K., Gonzales, E., Nichogiannopoulou, A., Wu, P., Nehen, S., and Georgopoulos, K. (1997) EMBO J. 16, 2004–2013
6. Kelley, C. M., Ikeda, T., Koipally, J., Avitahal, N., Georgopoulos, K., and Morgan, B. A. (1998) Curr. Biol. 8, 508–515
7. Hahm, K., Cobb, B. S., McCarty, A. S., Brown, K. E., Klug, C. A., Lee, R., Akashi, K., Weissman, I. L., Fisher, A. G., and Smale, S. T. (1998) Genes Dev. 12, 782–796
8. Winandy, S., Wu, P., and Georgopoulos, K. (1995) Cell 83, 289–299
9. Avitahal, N., Winandy, S., Friedrich, C., Jones, B., Ge, Y., and Georgopoulos, K. (1999) Immunity 10, 333–343
10. Wang, J.-H., Avitahl, N., Cambria, A., and Georgopoulos, K. (1999) Immunity 10, 543–553
11. Melnár, Á., and Georgopoulos, K. (1994) Mol. Cell. Biol. 14, 785–784
12. Sun, L., Huang, J., and Georgopoulos, K. (1999) EMBO J. 18, 5358–5369
13. Hahm, K., Ernst, P., Lo, K., Kim, G. S., Turck, C., and Smale, S. T. (1994) Mol. Cell. Biol. 14, 7111–7123
14. Wargnier, A., Lafaurie, C., Legros-Maida, S., Bourge, J. F., Sigaux, F., Sasportes, M., and Paul, P. (1998) J. Biol. Chem. 273, 35326–35331
15. Koipally, J., Renold, A., Kim, J., and Georgopoulos, K. (1999) EMBO J. 18, 396–400
16. Kim, J., Sif, S., Jones, B., Jackson, A., Koipally, J., Heller, B., Winandy, S., Veil, A., Sawyer, A., Ikeda, T., Kingston, R., and Georgopoulos, K. (1999) Immunity 10, 345–355
17. Zhang, Y., Sun, Z., W., Iratni, R., Erdjument-Bromage, H., Tempst, P., Hampsy, M., and Reinberg, D. (1998) Mol. Cell 1, 1021–1031
18. Tong, J., Hascic, C., Schnitzler, G. R., Kingston, R. A., and Schreiber, S. L. (1998) Nature 395, 917–921
19. Wade, P., Jones, P., Vermaak, D., and Wolfle, A. (1998) Current Biol. 8, 843–846
20. Struhl, K. (1996) Genes Dev. 12, 599–606
21. Laherty, C. D., Yang, W.-M., Sun, J.-M., Davie, J. R., Seto, E., and Eisenman, R. N. (1997) Cell 89, 349–356
22. Heinzle, T., Lavinsky, R. M., Muller, T., Soederstrom, M., Laherty, C. D., Worch, J., Brard, G., Ngo, S. D., Davie, J. R., Seto, E., Eisenman, R. N., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1997) Nature 387, 45–48
23. Sparrow, D., Miska, E., Langley, E., Reynaud-Dehau, S., Kotecha, S., Towers, N., Spohr, G., Kouzarides, T., and Mohun, T. (1999) EMBO J. 18, 5085–5096
24. Strouboulis, J., Damjanovski, S., Vermaak, D., Meric, F., and Wolffe, A. P. (1999) Mol. Cell. Biol. 19, 3958–3968
25. Luo, R., Postigu, A., and Dean, D. (1998) Cell 92, 463–473
26. Wong, C., and Privalsky, M. L. (1998) Mol. Cell. Biol. 18, 5500–5510
27. Mucsi, G. E. O., Burke, L. J., and Downes, M. (1998) Nucleic Acids Res. 26, 2899–2907
28. Ghosh, A., Steele, R., and Ray, R. (1999) Biochem. Biophys. Res. Commun. 260, 405–409
29. Lai, A., Lee, J. M., Yang, W. M., DeCaprio, J. A., Kaelin, W. G. J., Seto, E., and Branton, P. E. (1999) Mol. Cell. Biol. 19, 6632–6641
30. Boyd, J. M., Subramanian, T., Schaap, U., La Regina, M., Hayley, S., and Chimnadarai, G. (1993) EMBO J. 12, 469–478
31. Schaap, U., Boyd, J. M., Verma, S., Uhmann, E., Subramanian, T., and Chimnadarai, G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10467–10471
32. Turner, J., and Crossley, M. (1998) EMBO J. 17, 5129–5140
33. Katsanis, N., and Fisher, E. M. C. (1998) Genomics 47, 294–299
34. Sewalt, R. G. A. B., Gunster, M. J., van der Vlag, J., Stafijn, D. P., and Otto, A. P. (1999) Mol. Cell. Biol. 19, 777–787
35. Nibu, Y., Zhang, H., Bajer, E., Barolo, S., Small, S., and Levine, M. (1998) EMBO J. 17, 7009–7020
36. Sundqvist, A., Sallerbrant, K., and Svensson, C. (1998) FEBS Lett. 429, 183–188
37. Criqui-Filipe, P., Ducet, C., Mair, S. M., and Wasylyk, B. (1999) EMBO J. 18, 3392–403
38. Meloni, A., Smith, E., and Nevins, J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9574–9579
39. Poortinga, G., Watanabe, M., and Parkhurst, S. M. (1998) EMBO J. 17, 2067–2078
40. Brannen, J., Brown, J. D., Bates, R., Kimmel, D., and Moon, R. T. (1999) Development 126, 3159–3170
41. Fox, A. H., Liew, C., Holmes, C., Kowalski, K., Mackay, J., and Crossley, M. (1999) EMBO J. 18, 2812–2822
42. Li, S., Chen, P. L., Subramanian, T., Chinnadurai, G., Tomlinson, G., Osborne, C. K., Sharp, Z. D., and Lee, W. H. (1999) Curr. Biol. 9, 2067–2078
43. Nibu, Y., Zhang, H., Bajer, E., Barolo, S., Small, S., and Levine, M. (1998) EMBO J. 17, 7009–7020
44. Kolle, D., Brosch, G., Lechner, T., Lusser, A. A., and Loidl, P. (1998) Methods 15, 323–331
45. Sollerbrant, K., Chinnadurai, G., and Svensson, C. (1996) FEBS Lett. 429, 183–188
Ikaros Interactions with CtBP Reveal a Repression Mechanism That Is Independent of Histone Deacetylase Activity
Joseph Koipally and Katia Georgopoulos

J. Biol. Chem. 2000, 275:19594-19602.
doi: 10.1074/jbc.M000254200 originally published online April 13, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M000254200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 50 references, 24 of which can be accessed free at
http://www.jbc.org/content/275/26/19594.full.html#ref-list-1