Ikaros and Aiolos are Kruppel zinc finger proteins that play key roles in hemolymphoid development and homeostasis. We have previously shown that they can repress transcription through the recruitment of histone deacetylases (HDACs). Here, we provide the first functional evidence that these proteins can also repress gene function in a manner that does not require deacetylase activity. This functionality can be attributed in part to Ikaros interactions with the HDAC-independent corepressor, C-terminal binding protein (CtBP). However, mutations that block Ikaros-CtBP interactions do not abolish Ikaros's repression activity, implicating the involvement of additional corepressors. Consistent with this expectation, we show that Ikaros can interact with a CtBP-interacting protein (CtIP), which has also been linked to a deacetylase-independent strategy of repression. Despite being a CtBP interactor, CtIP's association with Ikaros does not require CtBP but instead relies upon its Rb interaction domain. Significantly, Ikaros can interact with Rb, which itself can repress gene function in a deacetylase-independent manner. A mutation in Ikaros that abrogates CtIP interactions significantly reduces repression, and a double mutation that prevents interaction with both CtIP and CtBP even further alleviates repression. Finally, we show that CtIP and CtBP can interact with the general transcription factors, TATA binding protein and transcription factor IIB, which suggests a possible mechanism for their deacetylase-independent mode of repression.

Ikaros is the founding member of a unique family of Kruppel zinc finger proteins that includes Aiolos, Helios, and Eos (1–5). The full-length members of this family are characterized by an N-terminal zinc finger DNA binding domain (6, 7) and two C-terminal zinc fingers that are involved in interactions with self and family members (8). Ikaros is comprised of seven exons from which at least eight isoforms can be generated through alternate splicing (Ik1 to 8). All Ikaros isoforms contain the C-terminal dimerization motif; however, they differ in the number of N-terminal DNA-binding zinc fingers (6, 7). Thus, Ikaros isoforms can be distinguished into two groups based on their DNA-binding ability. Genetic studies have clearly established that Ikaros proteins play critical roles during hemolymphopoiesis (9–11). Ikaros is required from the earliest stages of hemopoiesis, at the level of the hemopoietic stem cell (12), to the later stages of lymphoid cell fate determination; in addition, Ikaros proteins regulate lymphocyte proliferation and homeostasis (13, 14). Molecular and biochemical studies aimed at understanding the basis for these complex biological roles have revealed that Ikaros, in addition to functioning as an activator (15), can also potently repress gene expression (16).

Transcriptional repressors can be categorized in several ways. A common approach is to classify them as long range or short range repressors. Members of the former group, such as Groucho and Sir proteins, are capable of making a promoter resistant to all enhancers regardless of their distance from the promoter, whereas short range repressors, such as Kruppel and Giant, act in a less general manner to block the activity of locally bound activators (17). An alternative but non-mutually exclusive approach to repressor classification is based on the utilization, or the lack thereof, of the activity of histone deacetylase enzymes (HDACs) for repressor function. HDAC-mediated repression is expected to occur through the removal of acetyl groups from the N termini of histones, which presumably creates a compact chromatin configuration that inhibits transcription. Examples of HDAC-recruiting corepressors include the Sin3 and Mi-2β proteins (18, 19). Histone deacetylase-independent repressors are believed to function through multiple mechanisms, but the strategy that has been most extensively studied is the interaction of such factors with the basal transcriptional machinery; these interactions affect recruitment of the RNA Polymerase II holoenzyme to the promoter or events associated with promoter clearance and re-initiation (20–22). Examples of HDAC-independent corepressors include the C-terminal binding protein (CtBP) (23) and two CtBP interactors, the CtBP-interacting protein (CtIP) (24) and the histone deacetylase-related protein (HDAC/MTR) (25).

CtBP is a 48-kDa phosphoprotein that was first identified as an interactor of adenovirus E1A and more recently of several Dipteran and mammalian repressors (26, 27). Interaction between CtBP and these proteins, in most cases, is mediated through a PXL(S/R) motif (26, 27). The abbreviations used are: HD/HDAC, histone deacetylase; tk, thymidine kinase; AdML, adenovirus major late; AdMLP, AdML promoter; Ik, Ikaros; Aio, Aiolos; CtBP, C-terminal binding protein; CtIP, CtBP-interacting protein; Rb, retinoblastoma; BCoR, BCL6 corepressor; CoREST, repressor element silencing transcription corepressor; SMRT, silencing mediator of retinoic acid receptor transcription; aa, amino acid; DBD, DNA binding domain; GST, glutathione S-transferase; TFIIIB, transcription factor IIIB; TBP, TATA binding protein; CAT, chloramphenicol acetyltransferase; IP, immunoprecipitation; HDAC, histone deacetylase-related protein.
Ikaros as a Deacetylase Activity-independent Repressor

The transcriptional machinery. Through this pathway may involve interactions with the basal transcription machinery. We provide evidence to suggest that Ikaros repression of the adenovirus major late (AdML) promoter is insensitive to deacetylase inhibitor trichostatin A. Thus, we suggested that Ikaros mediates repression of this key regulator through the action of histone deacetylase inhibitors. We have previously shown that Ikaros can interact with the HDAC-recruiting factors, Sin3 and Mi-2 protein complexes have been described before (37). Antibodies used were: Myc-tag, FLAG, and GST (Sigma), Gal4, Sin3B (Santa Cruz Biotechnology), HDAC2 (Zymed Laboratories Inc.), Rb (Amersham Biosciences, Inc.), and anti-Ikaros and Mi-2, which have been previously described (37). CitP antibodies were generously provided by Dr. R. Baer and Dr. W.-H. Lee. Anti-HDRP was provided by Dr. X. Zhou and Dr. P. Marks.

RESULTS

Ikaros Repression of the tk Promoter Does Not Rely on Histone Deacetylase Activity—We have previously shown that Ikaros repression of the adenovirus major late (AdML) promoter is relieved in the presence of the histone deacetylase inhibitor, trichostatin A. Thus, we suggested that Ikaros mediates repression of this promoter through the action of histone deacetylase inhibitors. In addition to Ikaros, CtbP, Ik2BP, and Rb, that can work through a deacetylase-independent pathway. Mutations that abrogate CitP interactions reduce repression by Ikaros whereas those that prevent both CtbP and CitP associations even further alleviate repression. We provide evidence to suggest that Ikaros repression through this pathway may involve interactions with the basal transcriptional machinery.

EXPERIMENTAL PROCEDURES

Plasmids—BXG1, BXG1-Ik1, BXG1-Aio, BXG1-MAD, BXG1-nMAD, the reporters G5tkCAT and G5adMLPCAT, CDMS-Ik1, CDMS-HA-Ik1, CDMS-FLAG-Aio3, CDMS-FLAG-Helios, CDMS-FLAG-Eos (Daedalus), CDMS-MT-Sin3A, pCMV2-FLAGk1, pCMV2-FLAG Ik1, and GST-chIbp1 have been previously reported (38). Deletion and point mutants of Ik6 were generated by the Stratagene mutagenesis kit using Ik6 in the context of the BXG1 vector, which encodes the Gal4 DBD (amino acids 1–147) under the control of the SV40 promoter or in a CDMSFLAG vector. The following reagents were generously provided by the indicated investigators: GST-TFIB and pSG5-SMRT (Dr. M. Pivikas), MT-CoREST (Dr. G. Mandel), MT-Cabin1 (Dr. J. Liu), MT-BCoRest (Dr. V. Bardele), MT-Groucho (Dr. Y. Kim), MT-Ik6 and its CitP and Rb interaction mutants (Drs. A. Meloni and J. Nevins), an expression vector for Rb (Drs. T. Chen and Dr. J. Wang), and FLAG-HDRP (Dr. X. Zhou and Dr. P. Marks).

Transfections—293T and NIH3T3 cell lines were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (HyClone). Transfections of these cell lines were carried out using the HEPES buffered saline-CaPO4 method. For repression assays, 1 μg of the Gal4 fusion plasmid, 10 μg of the Gal4-reporter plasmid, and 0.5 μg of the pXGH5 growth hormone transfection efficiency control plasmid were used. Twenty-four hours after transfection cells were fed with fresh media, and 18–24 h later cells were harvested and processed for CAT assays as described previously (8). In those instances where the chimera A (Uptake Biotech) was employed, we added the drug doxorubicin to the cells 16–18 h before harvesting. Growth hormone assays were done as recommended by the manufacturer (Nichols Institute). Transfections were typically performed in duplicate and repeated between three and six times.
Ikaros as a Deacetylase Activity-independent Repressor

FIG. 1. Ikaros represses the tk promoter in an HDAC activity-independent manner. HDAC activity-independent repression by Ikaros. NIH3T3 cells transfected with 1 μg of the indicated Gal4 fusions (BXG1), 10 μg of G5adMLPCAT or G5tkCAT, and 1 μg of pXGH5 as a transfection efficiency control plasmid. 16–18 h before harvest, transfectants were treated (+Tricho) with trichostatin A (100 ng/ml) or left untreated (−Tricho). CAT activity was corrected for transfection efficiency using the growth hormone assay. This experiment was done in duplicate three times, and variation between experiments was less than 20%. Fold derepression upon trichostatin treatment is indicated below the graph and was calculated as the increase in corrected CAT activity upon trichostatin treatment divided by the corrected CAT activity in untreated cells. The left panel has been previously reported (16) and is included to allow comparison.

To identify other Ikaros-interacting factors that can account for its deacetylase-independent repression strategy, we screened a panel of seven well-established corepressors for binding the most full-length Ikaros isoform, Ik1, through a co-transfection/co-immunoprecipitation approach. These studies highlight the specificity that Ikaros shows in its interaction with histone deacetylases. This observation is highly reminiscent of the HDAC-independent repression by Ikaros. Therefore, CtIP can be recruited to Ikaros through CtBP, it can also be recruited through CtBP-independent means.

An Rb-binding Motif on CtIP Is Required for Its Interaction with Ikaros—Because both Ikaros and CtIP bind CtBP (24, 38), we tested whether the CtIP-Ikaros interaction was mediated through this common corepressor. Wild type Ikaros (Ik1) and a mutant form that cannot interact with CtBP (Ik1cm) were transfected along with CtIP and tested for binding. Interestingly, Ik1 and Ik1cm were capable of interacting with CtIP, albeit less strongly (Fig. 3C, compare IP lanes 1 and 4). Thus, although CtIP may be recruited to Ikaros through CtBP, it can also be recruited through CtBP-independent means.

In addition to interacting with CtBP, CtIP also interacts with the tumor suppressor and corepressor, Rb (24, 28). To determine whether CtIP might be recruited to Ikaros through an Rb-dependent mechanism, we co-transfected Ikaros with a mutant version of this domain that cannot interact with Sin3 (mMAD); the MAD protein serves as a positive control, because it has been shown to repress the tk promoter in a deacetylase-dependent manner (39) whereas its mutant variant serves as the negative control. Transfectants were either treated with trichostatin A or left untreated. CAT assays revealed that repression of the tk promoter by Gal4-Ik1 and Gal4-Aiolos, unlike Gal4-MAD, was not relieved over background levels in the presence of the deacetylase inhibitor (Fig. 1, right panel). Thus, Ikaros- and Aiolos-mediated repression of the tk, unlike the AdML-promoter, is not dependent on the activity of histone deacetylases. This observation is highly reminiscent of the corepressors Rb and HDRP, which also repress the tk promoter in a manner that does not rely on HDACs (40, 41). This provides the first functional evidence that Ikaros and Aiolos can repress transcription in a manner independent of HDAC activity.

Ikaros Can Interact with the HDAC Activity-independent Corepressor CtIP—Ikaros interactions with CtBP can account, in part, for this alternate repression strategy. Nevertheless, it is highly likely that other corepressors are also involved, because mutations that abolish the Ikaros-CtBP interaction still permit significant levels of repression (38). This supposition is further strengthened by the observation that Aiolos, which cannot interact with CtBP (38), continued to repress the tk promoter in the presence of the deacetylase inhibitor. To probe Western blots containing Ikaros complexes immunopurified from resting and cycling T lymphocytes, CtIP was found in complexes from both sources (Fig. 3B). Thus, CtIP appears to be an interactor of Ikaros proteins in lymphocytes.

An Rb-binding Motif on CtIP Is Required for Its Interaction with Ikaros—Because both Ikaros and CtIP bind CtBP (24, 38), we tested whether the CtIP-Ikaros interaction was mediated through this common corepressor. Wild type Ikaros (Ik1) and a mutant form that cannot interact with CtBP (Ik1cm) were transfected along with CtIP and tested for binding. Interestingly, Ik1 and Ik1cm were capable of interacting with CtIP, albeit less strongly (Fig. 3C, compare IP lanes 1 and 4). Thus, although CtIP may be recruited to Ikaros through CtBP, it can also be recruited through CtBP-independent means.

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Ikaros can bind the HDAC activity-independent corepressor CtIP through a CtBP-independent mechanism. A, interaction between CtIP and Ikaros isoforms (IkI, -2, -3, and -7) or exon 7 (E7) was tested by immunoprecipitation. B, in vivo interaction between CtIP and Ikaros in activated (a) and resting (r) T lymphocytes. Immunopurification of Ikaros-containing complexes was accomplished using a FLAGM2 column as previously described (37). The input (I), final wash (W), and eluate (E) were tested by immunoblot analysis using antibodies to CtIP, C, an Rb but not a CtBP motif on CtIP is critical for interactions with Ikaros. Ik1 wild type (Ik1WT) and Ik1 defective for interactions with CtBP (Ik1cm) were tested for their ability to interact with wild type CtIP. CtIP that cannot interact with CtBP (-CtBP), and CtIP that cannot interact with Rb (-Rb) by IP. The numbers below the immunoblot are included to aid the reader in comparing input and IP lanes. D, a summary of the interaction data obtained in C. E, C33A cells were transfected with Rb and FLAG-Ik1 to determine if the two proteins interact. To this end, whole cell lysates were immunoprecipitated with FLAG and an isotype control antibody. F, association between Ikaros family members and CtIP was tested by IP from whole cell extracts prepared from transfected 293T cells. The asterisks in A, E, and F identify the heavy chain of the immunoprecipitating antibody.

Mutations That Abolish Ikaros Associations with CtIP Alleviate Repression—To obtain CtIP interaction-defective Ikaros mutants, we targeted several mutations in exon 7 of the Ikaros isoform, Ik6 (Fig. 4B and data not shown). Of these mutants, M8, which contains a 20-amino acid deletion spanning residues 416–435, was found to significantly decrease CtIP binding (Fig. 4A, A and B). To determine the role of the CtIP-Ikaros interaction, we tested the effect of this mutation on repression by Ik6, the most potent repressor among the Ikaros isoforms. Like the CtBP interaction mutant (M1), the CtIP mutant (M8) caused a 50% reduction in repression of the tk promoter (Fig. 4B). When both these mutations were incorporated in a single molecule (M9), repression was further reduced to 15% of wild type Ik6 levels (Fig. 4B). These data indicate that CtIP and CtBP are major components of the deacetylase-independent repression strategy of Ikaros on the tk promoter.

CtIP Does Not Interact with HDAC2 and Precipitates Low Levels of HD Activity—What is the mechanism of CtIP-mediated repression? Although CtIP has been implicated in deacetylase-independent repression, little is known about its interactions with deacetylases. To determine whether CtIP can interact with endogenous histone deacetylases, we immunoprecipitated CtIP, and as a positive control Sin3A, from 293T cells. CtIP, unlike Sin3A, was not found associated with any significant amount of HDAC2 (Fig. 5A). This was verified by histone deacetylase assays of these immunoprecipitates, which indicated that HDAC activity associated with CtIP was close to background levels. In contrast, Sin3A, which associates with CtIP.

Wild type CtIP and CtIP mutants that are defective for interactions with Rb (Rb) and CtBP (-CtBP). Wild type CtIP interacted strongly with Ikaros whereas CtIP defective for interactions with CtBP showed a reduced interaction (Fig. 3C, compare IP lanes 1 and 2). Interestingly, CtIP that was defective for Rb interactions was significantly impaired in its interactions with Ikaros (Fig. 3C, compare IP lanes 1–3). Taken together, these data show that Ikaros can bind CtIP through a mechanism that relies upon an intact Rb binding domain on CtIP (summarized in Fig. 3D). So, can Ikaros associate with Rb? FLAG-Ik1 and Rb were co-transfected and tested for association by immunoprecipitation. Rb was indeed immunoprecipitated with Ikaros (Fig. 3E), which lends support to our earlier finding that CtIP interactions with Ikaros require a functional Rb interaction motif.

In summary, these data indicate that Ikaros interactions with CtIP do not require CtIP but instead require a functional Rb motif on CtIP.

CtIP Interacts with All Ikaros Family Members—Consistent with the finding that Ikaros does not require CtIP to interact with CtIP, the Ikaros family members, Helios, Aiolos, and Eos, which do not interact with CtBP, can bind CtIP (Fig. 3F). Further support for CtBP-independent recruitment of CtIP to Ikaros and its family comes from the finding that exon 7 of Ikaros, which lacks a CtBP-binding motif, can also interact with CtIP (Fig. 3A, lane 5). These data suggest that a region in exon 7 is most likely the CtBP-independent domain through which CtIP associates with Ikaros.

FIG. 3. Ikaros can bind the HDAC activity-independent corepressor CtIP through a CtBP-independent mechanism. A, interaction between CtIP and Ikaros isoforms (IkI, -2, -3, and -7) or exon 7 (E7) was tested by immunoprecipitation. B, in vivo interaction between CtIP and Ikaros in activated (a) and resting (r) T lymphocytes. Immunopurification of Ikaros-containing complexes was accomplished using a FLAGM2 column as previously described (37). The input (I), final wash (W), and eluate (E) were tested by immunoblot analysis using antibodies to CtIP, C, an Rb but not a CtBP motif on CtIP is critical for interactions with Ikaros. Ik1 wild type (Ik1WT) and Ik1 defective for interactions with CtBP (Ik1cm) were tested for their ability to interact with wild type CtIP. CtIP that cannot interact with CtBP (-CtBP), and CtIP that cannot interact with Rb (-Rb) by IP. The numbers below the immunoblot are included to aid the reader in comparing input and IP lanes. D, a summary of the interaction data obtained in C. E, C33A cells were transfected with Rb and FLAG-Ik1 to determine if the two proteins interact. To this end, whole cell lysates were immunoprecipitated with FLAG and an isotype control antibody. F, association between Ikaros family members and CtIP was tested by IP from whole cell extracts prepared from transfected 293T cells. The asterisks in A, E, and F identify the heavy chain of the immunoprecipitating antibody.
strongly with HDACs, supported 18-fold higher activity than background levels (Fig. 5A). These data lend support to the suggestion that CtIP likely utilizes HDAC-independent means to repress gene expression.

Ikaros, CtBP, and CtIP Interact with Components of the Basal Machinery—A well studied HDAC-independent repression strategy involves interactions with the basal transcriptional machinery that negatively affect pre-initiation complex assembly and/or promoter clearance (20, 22). Using GST fusions of different components of the basal transcriptional machinery, we found that in vitro translated CtIP can bind TFIIB (Fig. 5B) whereas the other deacetylase-independent Ikaros corepressor, CtBP, can associate with both the N (amino acids 1–128) and C termini of TBP as well as with TFIIB (Fig. 6A). Furthermore, Ikaros itself can interact with the C terminus of TBP (amino acids 128–328) and with TFIIB (Fig. 5B). Taken together these findings raise the possibility that the HDAC activity-independent repression mediated by Ikaros on the tk promoter may occur through interactions with components of the basal transcriptional machinery.

DISCUSSION

We have previously shown that Ikaros and Aiolos can repress transcription through the recruitment of histone deacetylases (16). In addition, Ikaros interacts with CtBP, which can repress through HDAC activity-independent mechanisms (38). On the basis of this interaction, we proposed that Ikaros might also be capable of this alternate repression strategy. Here, we provide the first functional evidence that Ikaros and Aiolos can effect repression in a manner that is independent of deacetylase activity. However, mutations in Ikaros that abolished interactions with CtBP could still repress transcription, indicating the involvement of other corepressors (38). Consistent with this expectation, we show that Ikaros interacts with the corepressors, CtIP and Rb, which are capable of deacetylase-independent repression. Finally, mutations that abrogate CtIP interactions with Ikaros alleviate repression, and those that prevent both CtBP and CtIP interactions even further reduce repression of the tk promoter.

Because both Ikaros and CtIP contain the CtBP penta-peptide interaction module and because both these factors can bind CtBP (24, 38), we considered the possibility that their association was mediated via CtBP. However, Ikaros proteins bearing mutations that prevented interactions with CtBP, were still able to interact with CtIP. Therefore, interaction with CtBP was not essential for CtIP associations with Ikaros. In support of this finding, a domain of Ikaros lacking a CtBP interaction
The recruitment of Ikaros to a promoter may be dictated by the promoter context. The corepressors that interact with Ikaros through Rb family proteins. In support of this finding, we have recently found that Ikaros can interact with another corepressor, histone deacetylase-related protein (HDRP) (data not shown). HDRP was first identified as an interactor of a key muscle regulatory protein, MEF2 (25), and was recently shown to bind CtBP and to repress the tk promoter in an HDAC-independent manner (41).

An enigma resulting from these studies is the basis for why repression of the tk versus the AdML promoters requires Ikaros to utilize two different repression strategies. If histone deacetylation is involved in repression through effecting DNA compaction, one would have expected HDAC recruitment to repress all promoters. One possible explanation for a promoter-selective function of HDACs is that the promoter context, defined by the other trans-acting factors bound to it, may only permit HDAC recruitment on a promoter like AdML but not one like tk. Thus, selective recruitment of co-factors by a DNA binding factor, influenced by its binding context, may underlie a transcription factor’s promoter-specific transcriptional functions.

Thus far, CtIP has been shown to interact with two tumor suppressors, BRCA1 (29, 34) and Rb (28). We have previously shown that dysregulation of Ikaros expression causes rapid development of leukemias and lymphomas (10). CtIP interactions with Ikaros may be involved, in part, in regulating the tumor suppressor function of Ikaros. The availability of the Ikaros-CtIP interaction mutant will allow this hypothesis to be tested. In conclusion, in this report we have presented several lines of evidence to show that Ikaros can function as a deacetylase-independent repressor in addition to its ability to repress through the recruitment of histone deacetylases (Fig. 6). This is a significant step forward in the attempt to molecularly dissect the workings of this key hemo-lymphoid regulator.

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