Induction of AP-2α Expression by Adenoviral Infection Involves Inactivation of the AP-2rep Transcriptional Corepressor CtBP1*

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AP-2 transcription factors execute important functions during embryonic development and malignant transformation. Recently, we have isolated a transcriptional repressor of AP-2α expression, the novel Krüppel-related zinc finger protein AP-2rep (Klf12). Here, we show that repression of AP-2α expression by AP-2rep is dependent on an N-terminal PVDSL motif that interacts specifically with the corepressor CtBP1 both in vivo and in vitro. This interaction motif was previously identified in the C-terminal region of the adenoviral oncoprotein E1A. Infection of both HeLa and PA-1 cells with adenovirus type 5 strongly induced AP-2α mRNA. Consistently, E1A was necessary and sufficient to mediate up-regulation of AP-2α. Transiently transfected wild-type E1A protein activated an AP-2rep sensitive cis-regulatory element of the AP-2α promoter, but E1A protein harboring a mutation in the PVDSL motif failed to activate. In summary, we conclude that the adenoviral oncoprotein E1A activates transcription from the endogenous AP-2α gene, an effect that involves transcriptional derepression of the AP-2α promoter by interaction of E1A with the AP-2rep corepressor CtBP1.

The basic helix-loop-helix transcription factor AP-2α represents the prototype of a small family of closely related and evolutionarily conserved DNA-binding proteins harboring helix-loop-helix dimerization motifs, AP-2α, AP-2β, and AP-2γ (1–3). Highly regulated expression patterns of AP-2α have been described during embryonic development of the neural tube, neural crest derivatives, eye and face, and limb bud, as well as urogenital and ectodermal tissues (4–6). Recently, germ line mutations in the human AP-2β gene have been shown to cause Char syndrome, a familial form of patent ductus arteriosus involving a specific defect in thoracic neural crest cells (7). Studies of AP-2-deficient mice reveal specific defects in the neural tube, craniofacial structures, and kidney, suggesting that AP-2 genes execute essential functions in regulating specific gene expression programs and cell survival during embryonic development (8–11). Consistently, a number of target genes that are transcriptionally activated or silenced in a cell type-specific fashion have been identified (12–14).

Specific expression patterns of AP-2α genes have further been implicated in malignant transformation and stress response of mammalian cells. AP-2α overexpression was found to result from and to be functionally involved in N-ras-mediated malignant transformation of PA-1 human teratocarcinoma cells (15). AP-2α and AP-2γ overexpression in breast cancer cells directly transactivation of the transmembrane tyrosinase-coupled receptor c-erbB2 (16) and correlate with regulation of multiple growth factor signaling pathways (17). A role of AP-2 in the mammalian stress response following ultraviolet A radiation has been identified (18). Specific interaction of AP-2 with the c-Myc-Max heterodimer negatively regulate c-Myc target genes and c-Myc-induced apoptotic cell death (19, 20). Therefore, it has been proposed that AP-2 genes are involved in programming cell survival, particularly in fast-proliferating cells under stress conditions or under conditions of limited external growth factor supply that occur particularly during embryonic development and in neoplastic tissues.

We have previously studied transcriptional mechanisms controlling the activity of the AP-2α promoter and identified a network of activating and silencing factors (20, 21). The novel Krüppel-related zinc-finger repressor factor AP-2rep (Klf12) mediated down-regulation of AP-2α expression (22). Interestingly, AP-2rep harbors a putative interaction domain for the transcriptional corepressor CtBP1, which also binds to the C terminus of the adenoviral oncoprotein E1A. Viral oncoproteins frequently reinstruct endogenous gene regulation and provide insights into molecular mechanisms of malignant transformation. In this study, we therefore investigated whether adenoviral infection elicits alterations in AP-2α expression patterns and explored the putative role of AP-2rep and CtBP1.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfections—HeLa and PA-1 clone 9117 (15) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Sigma). Transient transfections were performed using a standard calcium coprecipitation protocol as described previously (16). Luciferase activity was assayed as recommended by the manufacturer (Promega, Mannheim, Germany) in a Luminometer Lumat LB 9501 (Berthold, Wildbad, Germany). Relative light units were normalized to β-galactosidase activity and protein concentration. All experiments were repeated at least three times, with standard deviations less than 10%.

Reporter and Expression Plasmids—AP-2 promoter constructs have been described previously, as well as the cytomegalovirus promoter-driven AP-2α expression plasmid (21). The same cytomegalovirus promoter-driven vector (pCMX) vector was used to construct CtBP1 and E1A 13 S expression plasmids. The entire coding sequence of CtBP1 was amplified from 10 ng of plasmid DNA using the sense and reverse primers GGG GAA TTC TAT GC  and GGG GAA TTC CTA CAA CTA GTG ACT CG, respectively. The E1A 13 S sequence was amplified using the primers GGG GAA TTC CTA ATG AGA CAT ATT ATC TGC and GGG GAA TTC CTA ATG AGA CAT ATT ATC TGC.

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Induction of AP-2α Involves Inactivation of CtBP1

27945

TTT. The CtBP1 PCR fragment was digested with EcoRI, E1A was digested with HindIII, and both were ligated into pCMX-PL1 and verified by sequencing the entire open reading frame.

Site-specific Mutagenesis—The CtBP1-interacting motifs of AP-2α and E1A (PVDSLs and PLDSLs) were mutated to PVASS and PLASS, respectively, with the transformed plasmid used for transfection of HeLa cells as described in the manufacturer’s protocol (CLONTECH, Palo Alto, CA). The following primers were used: AP-2α mutant primer, GAG CCA GTG GCT AAT CTT CTG GAG; E1A mutation primer, CAA CCT TTG GCT AGC CTA ATT TGT TTT GTA GCC AGG AGC; hAP-2α mutant primer, GAG CCA GTG GCT AAT CTT CTG GAG; E1A mutation primer, CAA CCT TTG GCT AGC CTA ATT TGT TTT GTA GCC AGG AGC; hAP-2α mutation primer, GAG CCA GTG GCT AAT CTT CTG GAG; E1A reverse primer, CTA CGT CGC CCT GGA CTT CGA GC (21). Protein concentrations were estimated on a Coomassie-stained SDS-polyacrylamide gel and visualized using autoradiography.

Two-hybrid Screening and Two-hybrid Assays—The CLONTECH two-hybrid system was used according to the manufacturer’s instructions. A murine CtBP1 (day 17.5) brain cDNA library in the gal4DBD fusion vector, pGAD10, was screened in vitro translated protein was generated using T7 polymerase and the Promega TNT system. Glutathione S-transferase (GST) fusion proteins were purified as previously described (21). Protein concentrations were estimated on a Coomassie-stained SDS-polyacrylamide gel. Approximately equal amounts of GST fusion protein were mixed with [35S]labeled in vitro translated protein in HBBNP buffer (20 mM HEPES/HCl, pH 7.8, 50 mM KCl, 5 mM dithionine-labeled AP-2α and E1A selection primer, GTG TGC AGG ACT CAT CTA ATT TGT TTT GTA GCC AGG AGC; hAP-2α selection primer, GTG TGC AGG ACT CAT CTA ATT TGT TTT GTA GCC AGG AGC; H5 dl312 (lacking E1A) and H5 dl1135 (lacking amino acids 225–238 of E1A exon 2, including the PLDLS sequence at 233–237 (25)). The PCR was evaluated by melting curve analysis following the manufacturer’s instructions and checking the PCR products on 1.8% agarose gels. Each quantitative PCR was performed at least in duplicate.

RESULTS

The PVDSL Motif of AP-2α Rep Functions as a Transposable Repressor Domain—Conserved structural motifs in the transcriptional repressor AP-2α (KIF12) include three Krüppel-related zinc fingers and an H/C knuckle TGE(K/R)P(Y/F) in the C terminus and a serine/threonine-rich domain next to an PVDSL motif in the N-terminal region (Fig. 1A) (22). The consensus sequence P(V/L)DSLs, present in the adenosinone-coprotein E1A (27) and many mammalian transcriptional repressors, has been shown previously to recruit CtBP1 and CtBP2 corepressors (28, 29). To identify transposable protein domains eliciting transcriptional regulation, we constructed fusion proteins (schematically shown in Fig. 1A) of the AP-2α N-terminal region with the Gal4 DNA binding domain (Gal4/repNT), of the AP-2α C-terminal region with the Gal4 DNA binding domain (Gal4/repCT), and of the AP-2α C-terminal region with the viral activator VP16 (VP16/repCT), respectively. The effect of transiently transfected fusion proteins on the activity of appropriate luciferase reporters was assayed in HeLa cells and the human teratocarcinoma cell line PA-1 clone 9117 (Fig. 1B). As reporters we used TK-LUC plasmids containing three Gal4 binding sites (UAS) or two AP-2α binding sites (A32 (21)).

Transfection of Gal4/repNT, but not of the unmodified Gal4 protein, resulted in significant repression of the luciferase reporter (~10-fold).

Interestingly, transcriptional repression of the Gal4/repNT fusion protein was entirely abrogated by a double point mutation in the putative CtBP interaction motif (PVDSLs to PVASS). Transfection of a Gal4 protein fused with the AP-2α C-terminal region did not change activity of the Gal4 reporter, indicating that the zinc finger domain does not harbor any intrinsic transcription regulatory activity. As expected, fusion of the viral VP16 activator to the zinc finger domain resulted in strong activation of the AP-2α-dependent luciferase reporter. From these data, we concluded that the AP-2α N-terminal region harbors a transposable repressor activity, which is entirely dependent on the PVDSLs motif. Furthermore, the Krüppel-related zinc finger domain of AP-
2rep functions as a sequence-specific DNA binding domain without significant intrinsic transcriptional regulatory activity.

To address further whether the PVDLS motif conferred transcriptional repression we fused two copies of the wild-type and the mutated sequences (R_PVDLS_R and R_PVASS_R, respectively) with Gal4. Results from transient cotransfection with the Gal4-dependent luciferase reporter indicated that the putative CtBP interaction domain was both necessary and sufficient for transcriptional repression (Fig. 1C).

Recruitment of CtBP1 by AP-2rep Is Dependent on the PVDLS Motif—To demonstrate that the AP-2rep transcriptional repressor motif interacts with CtBP corepressors, we assayed protein-protein interaction. Screening 2 x 10^6 clones of a yeast two-hybrid library prepared from murine embryonal
brain at gestational stage day 17.5 with the AP-2α N terminus as a bait resulted in isolation of 71 independent clones. Determination of the respective cDNA inserts revealed that 65 of these clones contained partial or complete fragments of the murine CtBP1 cDNA and that the remaining six clones represented obvious artifacts (data not shown). A functional yeast two-hybrid assay indicated robust interaction that resulted in approximately one-third of β-gal reporter expression as compared with SV40 large T antigen interaction with p53, which was assayed for a positive control (Fig. 2A, pVA3/pTD1). Importantly, the mutated AP-2α protein, harboring the same two amino acid exchanges in the PVDLS motif as used for transient transfections in Fig. 1C, failed to interact with CtBP1.

To further assay in vitro interaction between AP-2α and CtBP1, we performed a GST pull down experiment. Therefore, GST fusion proteins containing either wild-type AP-2α or PVASS-mutated AP-2α were immobilized to glutathione-coupled Sepharose and incubated with in vitro translated 35S-labeled protein CtBP1 protein. As shown in Fig. 2B, CtBP1 was specifically retained by wild-type, but not by mutated AP-2α fusion protein. In control experiments, 35S-labeled CtBP1 also failed to interact with unmodified GST.

To verify specific protein-protein interaction of AP-2α with CtBP1 by a second independent method, we also performed coimmunoprecipitations both with in vitro translated AP-2α and CtBP1 proteins and with tagged AP-2α transiently expressed in HeLa cells. Therefore, we used a flag-modified AP-2α protein as a bait. As shown in Fig. 2C, we were able to coimmunoprecipitate specifically CtBP1 and also unmodified AP-2α protein.

When we transiently transfected FLAG-tagged AP-2α into HeLa cells, we were able to coprecipitate CtBP1 with an anti-FLAG antibody (Fig. 2D, lanes 5 and 6) from cell extracts. In comparison, we Western probed HeLa cell lysates transiently transfected with CtBP1 alone or with both CtBP1 and tagged AP-2α. Our results shown in Fig. 2D (lanes 1–3) clearly show that a significant portion of endogenous CtBP-1 present in HeLa cells can be coimmunoprecipitated with the tagged AP-2α protein, indicating tight interaction between the two proteins. In summary, we concluded from these data that the transcriptional repressor AP-2α recruits the corepressor CtBP1 via physical interaction through the PVDLS domain.

**Fig. 2. Interaction of AP-2α and CtBP-1 dependent on the PVDLS domain.** A, yeast two-hybrid assay. β-Galactosidase activity mediated by p53/SV40-large T interaction (pVA3/pTD1) was assayed as a positive control. As bait, wild-type AP-2α NT (as shown in Fig. 1A) or AP-2α NT mutated in the PVDLS motif was coexpressed with CtBP-1. B, GST in vitro binding assay. Immobilized GST-AP-2α fusion protein retained 35S-labeled CtBP1, but fusion protein mutated in the PVDLS motif failed to interact. C, immunoprecipitation in vitro. Unlabeled AP-2α/FLAG protein was coimmunoprecipitated with 35S-labeled AP-2α, AP-2α/FLAG, CtBP1, or AP-2α and immunoprecipitated. Coprecipitated proteins were visualized by autoradiography. AP-2α (413 amino acids), AP-2α/FLAG (418 amino acids), and CtBP1 (440 amino acids) run at almost identical relative molecular masses of ~50 kDa. D, CtBP1 immunostaining of Western blots prepared from FLAG-immunoprecipitate of HeLa cells transfected with FLAG-AP-2α or CtBP1. Transfections were performed as follows: lane 1, CtBP1; lanes 2 and 3, FLAG-AP-2α and CtBP1; lanes 5 and 6, FLAG-AP-2α. Controls were as follows: lane 1, extract of HeLa cells transfected with CtBP1 (without immunoprecipitation); lane 4, no protein extract; lane 7, immunoprecipitation from HeLa extracts in the absence of transfected FLAG-AP-2α.
To analyze whether induction of AP-2 protein by adenoviral infection involved up-regulation of AP-2α mRNA, we performed quantitative real-time reverse transcription-PCR on RNA templates extracted from HeLa cell cultures infected for 24 h (Fig. 3C). The ratio of AP-2α versus β-actin mRNA was increased ~8-fold by wild-type adenovirus but was not significantly altered after infection with identical titers of mutant adenovirus strains (50 PFU/cell) or in mock-infected cells. Further controls showed that the induction of AP-2 was dependent on the multiplicity of infection. Infection with a 1/10 titer of wild-type virus (5 PFU/cell) resulted in ~5-fold AP-2α induction, and infection with a 1/100 titer (0.5 PFU/cell) resulted only in 1.5-fold induction (data not shown). From these experiments, we inferred that induction of AP-2α by adenoviral infection is critically dependent on the intact E1A-CtBP1 interaction motif and involves both AP-2α mRNA and protein.

Therefore, we investigated the effect of transiently expressed E1A protein and explored the role of an intact CtBP interaction motif in regulation of AP-2α expression. In parallel, we measured the effect on a luciferase reporter under control of the dimerized A32 cis-regulatory element of the AP-2α promoter (Fig. 4A) and on endogenous AP-2α protein levels quantified by Western blots (Fig. 4B). A transient transfection of E1A resulted in ~2-fold activation of the A32-dependent luciferase reporter. Importantly, an E1A construct harboring the double point mutation in the CtBP interaction motif did not activate the luciferase reporter. In contrast, we observed moderate but reproducible repression to ~0.6-fold activity (Fig. 4A, E1A mut). Consistent with these results, transcriptional repression of the A32-dependent reporter by AP-2rep was entirely abrogated by the double point mutation in the PVDLS interaction domain. Again, we observed reproducible activation of the reporter, possibly due to interference with endogenous wild-type AP-2rep repressor protein and to mutually exclusive binding with activating transcription factors such as BTEB-1 (21). Importantly, transiently expressed CtBP1 protein

![Figure 3: Induction of AP-2α expression by adenovirus type 5 infection.](image)

**Figure 3.** Induction of AP-2α expression by adenovirus type 5 infection. **A,** quantitation of AP-2α and E1A on Western blots prepared from cell lysates of HeLa (left) and PA-1 9117 cells (right) infected with adenovirus-5. B, AP-2α and β-actin expression in HeLa cells infected for 24 h with adenovirus type 5, with mutant 312 (E1A deletion) and mutant 1135 (microdeletion in the E1A-CtBP interaction motif) in comparison to mock-infected cells. Infection was performed with an identical titer of all three adenoviral strains (50 PFU/cell). C, measurement of AP-2α mRNA by quantitative real-time PCR (LightCycler) in wild-type (Ad5), mock, mutant 312 (E1A deletion), and mutant 1135 (microdeletion in the E1A-CtBP interaction motif) infected HeLa cells. Infection was performed with 50 PFU/cell of all three adenoviral strains. Bars indicate the ratio of AP-2α mRNA versus β-actin mRNA.

![Figure 4: Activation of AP-2α by E1A and repression by AP-2rep dependent on the CtBP1 interaction domain.](image)

**Figure 4.** Activation of AP-2α by E1A and repression by AP-2rep dependent on the CtBP1 interaction domain. A, activity of the AP-2rep-sensitive A32-TK-luciferase reporter in HeLa cells transiently transfected with wild-type E1A, E1A mutated in the PLDLS motif, wild-type AP-2rep, and AP-2rep mutated in the PVDLS motif or CtBP1. As a negative control, cells were mock-transfected with the empty pBluescript SKII-vector. B, corresponding Western blot of HeLa cells transfected as shown in A reveals regulation of endogenous AP-2α expression by AP-2rep, E1A, and CtBP1.
also repressed the A32 luciferase reporter, indicating that CtBP1 elicits intrinsic transcriptional repressor activity when appropriately recruited. All results obtained from measuring luciferase reporter activities were closely paralleled by changes of endogenous AP-2α protein visualized by Western blots of HeLa cell extracts (Fig. 4B). In general, the effects of transient transfections were significantly smaller than changes in AP-2 expression by adenoviral infections, because we observed much higher E1A expression levels after infections (data not shown), and the activity of an AP-2-dependent promoter depends also on AP-2 isoforms other than AP-2α.

Taken together, our data provide evidence that both adenoviral infection and transfection of the adenoviral oncoprotein E1A activate expression of the endogenous AP-2α gene. Furthermore, activation of AP-2α transcription is dependent on the presence of a functional E1A-CtBP interaction motif and involves inactivation of the transcriptional silencer AP-2rep by direct interaction with the corepressor CtBP1.

**DISCUSSION**

Recently, we identified the A32 element, an important cis-regulatory element in the AP-2α promoter mediating both activation and repression of AP-2α mRNA transcription. Promoter activity was silenced through sequence-specific binding of AP-2rep, a novel Krüppel-related zinc finger repressor, which interacted mutually exclusive with activating factors (21). In the present study, we provide further evidence for a role of AP-2rep in transcriptional repression that involves physical association with the corepressor CtBP1. Data presented in Figs. 1 and 2 show that the transcriptional repressor activity of AP-2rep is tightly associated with a functional CtBP interaction motif and demonstrate strong interaction of the two proteins by coimmunoprecipitation both from endogenous cell extracts and in vitro. Therefore, specific interaction with CtBP1 through the PVDLS domain is functionally important and identifies AP-2α as a novel target gene of CtBP-mediated repression.

The function of CtBP1 and its close homolog CtBP2 as transcriptional corepressors for the zinc finger-transcription factor E1A has been previously described by Furusawa et al. (29). Interestingly, these authors have described specific embryonic expression patterns of different AP-2 genes during development of the face, cephalic ganglia, dorsal root ganglia, and limb. There-
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