The adenovirus 12 E1A proteins can bind directly to proteins of the p300 transcription co-activator family, including the CREB-binding protein CBP and p300

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The cellular transcription co-activators p300 and the CREB-binding protein CBP are cellular targets for transformation by the E1A proteins of non-oncogenic adenovirus 5 (Ad5). In this study, we show that the E1A proteins of oncogenic Ad12, like those of Ad5, can also bind to CBP and that this interaction is direct. In addition, we show that the Ad12 E1A proteins can also bind directly to p300. These results suggest that E1A can modulate the function of proteins of the p300 family via direct protein–protein interactions.

The CREB-binding protein CBP, together with the closely related protein p300, belong to a conserved family of transcription co-activators (Arany et al., 1994). Co-activators are a new class of transcriptional activators that are required at many promoters and do not bind directly to DNA (Guarente, 1995). Both CBP and p300 bind to the N-terminal domain of the transforming E1A proteins of non-oncogenic human adenovirus 5 (Ad5) and these interactions are important for Ad5 E1A-induced cell immortalization, transformation and repression of neuronal differentiation (Boulukos & Ziff, 1993; for reviews see Peep & Zantema, 1993; Moran, 1994; Hagmeyer et al., 1995). Studies using hybrid Ad5–Ad12 E1A genes have indicated that sequences in the non-conserved N-terminal domain of the E1A proteins may also contribute to differences in the tumorigenic potential of non-oncogenic Ad5 and oncogenic Ad12 (Jelinek et al., 1994). Recently, the loss of CBP gene function has been associated with Rubinstein–Taybi syndrome (RTS). RTS constitutes one of the first clear-cut examples of a mental retardation syndrome linked to an abnormality in gene transcription (Petrij et al., 1995). Accordingly, CBP may play an important role not only in gene regulation, but also in cell-cycle control and differentiation. Perturbation of CBP function by viruses or genetic mutation may therefore have profound pathological consequences.

Studies on CBP and p300 thus represent a remarkable convergence of the fields of gene regulation, virology and human genetics. Analysis of the interplay of proteins of the p300 family with E1A proteins of different Ad serotypes may provide important insights into the molecular mechanisms of (oncogenic) transformation and concomitant alterations in gene regulation by virus proteins (for review see Hagmeyer et al., 1995).

In this study, we investigated whether the E1A proteins of oncogenic Ad12, like the non-oncogenic Ad5 E1A proteins, can bind to CBP. In addition, we performed Far Western bloting to establish whether the E1A proteins can bind directly to CBP and p300.

To analyse systematically the interactions of cellular proteins with Ad5 and Ad12 E1A proteins, we generated a panel of plasmids coding for E1A–glutathione S-transferase (GST) fusion proteins. One of these, pGSTK-12E1A-1, codes for a product consisting of GST–Ad12 E1A-N-CR1-CR2 (Fig. 1). First, using GST ‘pull-down’ assays followed by Western

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Fig. 1. (a) The Ad12 E1A proteins. The regions preserved between human Ad5, Ad7 and Ad12 and simian adenovirus SA7 are shown (CR1, CR2 and CR3). The 12S product lacks the CR3 region. (b) Plasmid pGSTK-12E1A-1. Sequences of Ad12 E1A coding for the non-conserved N-terminal domain, CR1 and CR2 were inserted into a derivative of the pGEX vectors (Smith & Johnson, 1988), pPR269. For labelling purposes, an oligonucleotide encoding a recognition site for cAMP-dependent kinase of heart muscle (HMK) was cloned downstream from the GST gene. The sequence of this peptide recognition site is indicated.
Recently, we showed that GST–Ad5 E1A and GST–Ad12 E1A fusion proteins, including GSTK-12E1A-1, showed the expected binding behaviour with respect to already identified factors such as the retinoblastoma gene product (Rb), cyclin-dependent kinase 2 (cdk2) and cyclin A (data not shown). In addition, in GST pull-down experiments with [35S]methionine-labelled cell extracts we found that fusion proteins carrying the N-terminal domain and CR1 of the Ad5 or Ad12 E1A proteins bound cellular proteins in the 300 kDa range (data not shown; Wang et al., 1993). Subsequently, we combined a GST pull-down assay with Western blot analysis to test whether the Ad12 E1A protein could bind CBP. Briefly, an extract was prepared from HeLa cells in a non-stringent buffer (E1A lysis buffer; Dorsman et al., 1995). This extract was incubated with glutathione beads carrying GST or GSTK-12E1A-1. Equal amounts of each fusion protein were employed in each reaction, as judged from Coomassie blue staining. After incubation for 4 h, the complexes were collected, washed four times with E1A lysis buffer and resolved by SDS–PAGE. After electrophoresis, the proteins were blotted using the procedure for wet electrophoretic transfer of high molecular mass proteins (Harlow & Lane, 1988). Enhanced chemiluminescence (ECL) detection of the relevant antigens was performed according to the instructions of the manufacturer (Amersham). For the detection of CBP, rabbit polyclonal antibody A-22, which specifically recognizes CBP, was used (Santa Cruz Biotechnology; Chirivia et al., 1993; Lundblad et al., 1995). Rabbit polyclonal antibody CBD, which recognizes both p300 and CBP (a kind gift from R.H. Goodman, Vollum Institute, Portland, USA), and mouse monoclonal antibody NM11, which had been raised against p300 but also recognizes CBP (E. Moran, Fels Institute for Cancer Research and Molecular Biology, Philadelphia, USA, personal communication; Pharmingen), were used in addition. The results of these experiment are shown in Fig. 2. The CBP polypeptide has a predicted molecular mass of 265 kDa, but in the cell types we investigated, including HeLa cells, we did not observe any difference in migration pattern between CBP and p300 (Fig. 2a). For ECL detection of the associating proteins, antibodies A-22 and NM11 were used (Fig. 2b, c). With both A-22 and NM11 a clear signal was observed at the position of the 300 kDa protein when GSTK-12E1A-1 was used in a pull-down experiment (Fig. 2b, c). No signals were seen when GST alone was used. Use of the Ad5 E1A–GST fusion protein yielded essentially the same results (data not shown). Furthermore, in vitro-translated CBP also bound to Ad5 and Ad12 E1A in pull-down assays (data not shown). We conclude that the Ad12 E1A proteins, like the Ad5 E1A proteins, bind CBP.

Proteins detected in the GST pull-down assays and co-immunoprecipitation studies may have bound to E1A either directly or indirectly. To clarify whether members of the p300 family bind directly to the E1A proteins, we combined GST pull-down and co-immunoprecipitation experiments with Far Western blotting. Briefly, E1A proteins plus associating proteins were precipitated and the complexes fractionated on SDS–polyacrylamide gels. The proteins were blotted and the filter was probed with purified and labelled GSTK-12E1A-1 (Fig. 1b). The procedures for labelling of the fusion protein, blotting, incubation and washing were essentially the same as those described previously (Kaelin et al., 1992). The results are shown in Fig. 3 (a, b). In a GST pull-down experiment with the Ad12 E1A fusion protein, one strongly reactive band was observed, migrating at the approximate position of a 300 kDa protein (Fig. 3a). This band was also observed clearly using whole cell extract but was absent in the experiment with GST alone (Fig. 3a) and when a purified and labelled GST protein was used as the probe (data not shown). Therefore, we concluded the possibility that the cellular protein reacting with the probe was a GST-binding protein. Prominent bands at positions of other proteins were not observed. It has been reported that when Rb is over-expressed it can bind directly to Ad5 E1A (Honkisz et al., 1991). However, under our conditions, which did not employ over-expressed Rb, such an interaction was not seen. In addition, we performed co-immunoprecipitation experiments on extracts in E1A lysis buffer prepared from baby rat kidney cells transformed by Ad12 E1 (RIC cells) and thus expressing Ad12 E1A proteins. For these cells, rabbit polyclonal antibody p300, raised against

Fig. 2. Analysis of the interaction of Ad12 E1A with CBP. (a) Western blot analysis of whole HeLa cell extracts (WCE) using antibodies directed against members of the p300 family. Proteins were fractionated on a 6% SDS–polyacrylamide gel, blotted and detected by the ECL method. The following antibodies were used: lane 1, rabbit polyclonal antibody A-22, which specifically recognizes CBP; lane 2, rabbit polyclonal antibody CBD, which recognizes both CBP and p300; lane 3, mouse monoclonal antibody NM11, which also recognizes both CBP and p300. (b) GST–Ad12 E1A pull-down experiments. Beads carrying GST or a GSTK-12E1A-1 were incubated with HeLa cell extract. Proteins were fractionated on an 8% SDS–polyacrylamide gel. Associating proteins were identified by Western blotting using antibody A-22. Lane 1, whole (HeLa) cell extract (WCE); lane 2, GST pull-down; lane 3, GST–E1A pull-down. (c) The same experiments as described in (b) were performed but the associating proteins were detected with antibody NM11.
Ad12 E1A–CBP/p300 interaction

Fig. 3. Far Western blot analysis of the interaction of Ad12 E1A with CBP and p300. The position of the 200 kDa standard is indicated. (a) GST–Ad12 E1A pull-down experiments. Beads carrying GST or GSTK–12E1A-1 were incubated with HeLa cell extract and protein complexes were separated on a 6% SDS–polyacrylamide gel. Associating proteins were detected in a Far Western blot using purified and labelled GSTK–12E1A-1 as a probe. Lane 1, whole (HeLa) cell extract (WCE); lane 2, GST pull-down; lane 3, GST–E1A pull-down. (b) Ad12 E1A co-immunoprecipitation experiments. E1A plus associating proteins were precipitated from baby rat kidney cells expressing the wild-type Ad12 E1A proteins (RIC cells). Proteins were separated and detected as described in (a). Lane 1, whole RIC cell extract (WCE); lane 2, precipitation with non-immune serum (NI); lane 3, precipitation with polyclonal antibody p30 against Ad12 E1A. (c) Analysis of Ad12 E1A–CBP interaction. Proteins were precipitated from HeLa cell extracts with antibodies raised against members of the p300 family and were separated and detected as described in (a). Lane 1, whole HeLa cell extract (WCE); lane 2, precipitation with monoclonal antibody NM11 raised against p300; lane 3, precipitation with polyclonal antibody A-22 against CBP, which specifically recognizes CBP; lane 4, precipitation with polyclonal antibody CBD raised against CBP. (d) Analysis of Ad12 E1A–p300 interaction. A HA-tagged p300 protein was precipitated from C33A cells transfected with a vector expressing the human p300 cDNA. Proteins were separated and detected as described in (a). Lane 1, C33A cell extract (WCE); lane 2, precipitation with a control monoclonal antibody (C); lane 3, precipitation with monoclonal antibody 12CA5.

sequences in the C-terminal domain of the Ad12 E1A protein, was used (Peeper et al., 1992). One strong band was observed at the approximate position of p300 (Fig. 3b). This band was absent when proteins were precipitated with the non-immune control antibody (Fig. 3b). Apparently, the cellular protein that interacts strongly and specifically with the probe binds to the Ad12 E1A proteins both in vitro and in vivo. Analogous results were obtained when the Ad5 E1A proteins were used (data not shown). It is therefore highly probable that the observed direct interaction was caused by binding of Ad12 E1A to p300 and/or CBP. To test this, we performed immunoprecipitations with a panel of antibodies raised against CBP or p300. The immunoprecipitated proteins were tested in a Far Western blot, again with GSTK–12E1A-1 as the probe. A strong signal was observed in immunoprecipitations with all three antibodies (Fig. 3c). In immunoprecipitations with control antibodies, these bands were absent (data not shown). Taking into account that the polyclonal antibody A-22 is specific for CBP, these results suggest that the N-terminal domain and CR1 of the Ad12 E1A proteins can interact directly and specifically with CBP and probably also with other members of the p300 co-activator family.

Antibodies NM11 and CBD detect both CBP and p300 and for this reason could not be used to determine whether Ad12 E1A bound directly to p300. To determine whether Ad12 E1A indeed displays this capacity, we combined transient expression experiments with Far Western analysis. A vector expressing human p300 tagged with haemagglutinin (HA) (Eckner et al., 1994) was transfected into C33A cells by the calcium phosphate technique (Graham & van der Eb, 1973). After 48 h, lysates were prepared and precipitated with mouse monoclonal antibody 12CA5, which recognizes the HA tag, and with a control monoclonal antibody. The precipitates were tested in both Western and Far Western blots. The Western blot analysis indicated that a protein of 300 kDa was precipitated with antibody 12CA5 and not with the control antibody (data not shown). In a Far Western blot this protein could also be easily detected (Fig. 3d). These data show that Ad12 E1A can also bind directly to p300.

Various models have been proposed for the interplay between the Ad5 E1A proteins and members of the p300 family and how these interactions affect gene-regulatory events and transformation (for a recent review see Hagmeyer et al., 1995). Both CBP and p300 can bind to a variety of enhancer-binding factors, including the AP1/ATF families, and members c-Jun and CREB, which have critical functions in several important biological processes such as cell proliferation, differentiation and apoptosis (Chrivita et al., 1993; Arias et al., 1994; Kwok et al., 1994; Arany et al., 1995). Therefore, it seems probable that the interplay of the E1A proteins with co-activators of the p300 family and transcription factors that bind p300 and/or CBP may play an important role in (oncogenic) transformation. The E1A proteins may repress transcription of some p300- and AP1/ATF-regulated genes by sequestering an active co-activator from the promoter context by direct binding. The finding that E1A can bind directly to p300 and CBP supports this model. In addition, differences in the way the Ad5 and Ad12 E1A proteins interact with members of the p300 and AP1/ATF families could contribute to their differences in transforming and oncogenic potential. In agreement with this idea, the Ad5 and Ad12 E1A proteins have different effects on the expression levels of various genes coding for proteins of the Jun family (van Dam et al., 1990; Meijer et al., 1991). Accordingly, Ad12-transformed cells
contain a different repertoire of AP1 proteins to Ad5-transformed cells, with the concomitant effects on the regulation of target genes. A further systematic analysis of the members of the p300 and AP1/ATF families and of their interplay with E1A proteins of different serotypes, coupled to a search for biologically relevant p300- and CBP-regulated target genes, might lead to a better understanding of (oncogenic) transformation.

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