Topors, a p53 and topoisomerase I binding protein, interacts with the adeno-associated virus (AAV-2) Rep78/68 proteins and enhances AAV-2 gene expression

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The adeno-associated virus type 2 (AAV-2) Rep proteins are essential for AAV DNA replication and regulation of AAV gene expression. We have identified a cellular protein interacting with Rep78 and Rep68 in yeast two-hybrid analysis and in GST pull-down assays. This protein has recently been described as both a p53 (p53BP3) and a topoisomerase I interacting protein (Topors). It contains an arginine/serine-rich domain, a RING finger domain and five PEST sequences. A minimal sequence sufficient for interaction with Rep was mapped to Topors amino acids 871 to 917. We show that the same region is also involved in the interaction with p53. Rep sequences involved in interaction with Topors were mapped to Rep amino acids 172 to 481. Overexpression of Topors stimulated AAV gene expression in the absence of helper virus, suggesting a function of Topors as a transcriptional regulator.

Adeno-associated virus type 2 (AAV-2) is a human parvovirus that, for efficient reproduction, requires coinfection of its host cell with a helper virus such as adenovirus or herpesvirus (Bems & Linden, 1995). The 4.7 kb single-stranded DNA genome (Srivastava et al., 1983) consists of two open reading frames (ORFs) flanked by two 145 base pair inverted terminal repeats (ITRs). The ORF in the right half of the AAV genome encodes the three structural proteins, VP1, VP2 and VP3, which are transcribed from the p40 promoter. The ORF in the left half of the genome encodes four overlapping non-structural proteins, termed Rep (Mendelson et al., 1986). The p5 promoter directs the synthesis of Rep78 and Rep68, while the p19 promoter drives the expression of Rep52 and Rep40. Rep78 and Rep68 are essential for AAV DNA replication (Tratschin et al., 1984) and regulation of AAV gene expression (Labow et al., 1986; Tratschin et al., 1986). They possess ATP-

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Fig. 2. Mutational analysis of the Rep–Topors and p53–Topors interactions in the yeast two-hybrid system using a CPRG-based liquid culture β-galactosidase assay. (A) Different parts of the Rep coding region were fused in-frame to the Gal4 DNA binding domain in pGBT9 and cotransformed into yeast SFY526 cells together with Topors amino acids 854 to 1045 fused to the Gal4 transactivation domain in pGAD424. The Rep sequences fused to the Gal4 DNA binding domain are shown schematically on the left. The respective β-galactosidase activities expressed in units are shown on the right. (B, C) Different parts of the Topors protein, shown schematically on the left, were fused in-frame to the Gal4 transactivation domain in pGAD424 and
dependent helicase, site- and strand-specific endonuclease and sequence-specific DNA binding activities required for AAV-2 DNA replication (Im & Muzyczka, 1990). In the absence of a helper virus, Rep78 and Rep68 negatively regulate the AAV-2 promoters p5, p19 and p40 (Beaton et al., 1989; Kyostio et al., 1994; Trempe & Carter, 1988). In the presence of adenovirus, Rep78 and Rep68 activate the p19 and p40 promoters. This activation depends on Rep binding sites in the ITRs (Pereira et al., 1997; Weger et al., 1997) and in the p5 promoter (Pereira et al., 1997) as well as additional elements in the p19 and p40 promoters (McCarty et al., 1991; Pereira & Muzyczka, 1997a, b). The Rep proteins also downregulate a variety of heterologous viral and cellular promoters (Hermonat, 1994; Horer et al., 1995; Labow et al., 1987), inhibit cellular and viral DNA replication (Hermonat, 1989; Yang et al., 1995) and suppress cellular transformation by cellular and viral oncogenes (Hermonat, 1989; Khleif et al., 1991). Whether these activities contribute to the onco-suppressive properties of AAV observed in mice (de la Maza & Carter, 1981) is not clear. A recent report provided evidence that the single-stranded AAV genome induces apoptosis in p53-deficient cells in the absence of AAV gene expression (Raj et al., 2001). So far few relevant targets for the diverse Rep activities have been identified. Specific cis-regulatory elements that bind the AAV-2 Rep78 and Rep68 proteins have been identified in the AAV-2 p5 promoter, the human immunodeficiency virus LTR and the H-ras promoter (Batchu & Hermonat, 1995; Batchu et al., 1994; Kyostio et al., 1995). Protein–protein interactions with Rep have been described for SPI (Pereira & Muzyczka, 1997b), the high mobility group chromosomal protein 1 (Costello et al., 1997), the TATA box binding protein (Hermonat et al., 1998), p53 (Batchu et al., 1999), the transcriptional coactivator PC4 (Muramatsu et al., 1998; Weger et al., 1999) and the protein kinases PrKX and PKA (Chiorini et al., 1998; Di Pasquale & Stacey, 1998).

To identify additional cellular Rep targets relevant for the diverse effects of Rep78 and Rep68 we employed the yeast two-hybrid system as outlined in Weger et al. (1999). The majority of Rep68 interaction partners corresponded to the transcriptional coactivator PC4 (Weger et al., 1999). To identify Rep-interacting cellular proteins that do not correspond to PC4, positive clones obtained from a yeast two-hybrid screen (5 × 10⁶ yeast transformants) of a HeLa cDNA library (1 × 10⁶ independent clones) with the central part of the large AAV-2 Rep proteins (pGBT9–M172/530; Fig. 1A) as bait were subjected to colony hybridization with a PC4 hybridization probe. Three PC4-negative clones were identified, which were positive for interaction with pGBT9–Rep68 and negative with unrelated bait proteins after retransformation. The coding region of one of these clones corresponded to the C-terminal 192 amino acids of a protein recently identified both as a novel p53 binding protein, named p53BP3 (Zhou et al., 1999), and as a DNA topoisomerase I binding protein, named Topors (Haluska et al., 1999), in yeast two-hybrid screens. Sequence analysis had predicted an ORF of 815 amino acids for p53BP3 (Zhou et al., 1999) and an ORF with 230 additional amino acids at the N terminus for Topors (Haluska et al., 1999). We cloned the entire cDNA sequence by RT–PCR from HeLa mRNA and obtained nucleotide sequence data corresponding to that published for Topors (Haluska et al., 1999). Thus we will refer to this protein as Topors. To demonstrate direct binding of Rep to the C terminus of Topors, a GST fusion protein encoding the 192 C-terminal amino acids of Topors was expressed in E. coli. The GST–Topors fusion protein and GST alone as a control were purified on glutathione–Sepharose beads and incubated with in vitro-transcribed/translated and $^{35}$S-labelled Rep78 or Rep52, respectively, as described in Weger et al. (1999). Rep78 (Fig. 1B, left panel), but not Rep52 (Fig. 1B, right panel), was specifically retained by the GST–Topors(854–1045) fusion protein. These findings demonstrate binding of the large AAV-2 Rep proteins to the C terminus of Topors both in vivo and in vitro.

The function of Topors is as yet unknown. The protein sequence contains a RING-type zinc finger domain, a bipartite nuclear localization signal and a region rich in arginine–serine dipeptides (RS domain) (Fig. 1C). In addition, Topors features five stretches of amino acids enriched in proline, glutamine, serine and threonine (PEST sequences). PEST sequences have been shown to be a characteristic of several rapidly degraded proteins (Rechsteiner & Rogers, 1996). The presence of an RS domain suggests that Topors might be involved in mRNA splicing. However, Topors does not contain a consensus RNA binding domain, which has been found in most of the mRNA splicing factors examined so far (Fu, 1995; Zahler et al., 1992). The RING domain of Topors is closely related to similar domains in the ICP0 family of herpesvirus immediate early transactivators. These proteins are implicated in the regulation of viral gene expression and the reactivation of latent herpesvirus, with the RING domain required for these functions (Everett et al., 1995; Lium & Silverstein, 1997). Increasing evidence has been gathered to show that many proteins containing the RING domain may function as E3 ubiquitin ligases regulating proteasome-dependent degradation of cellular proteins (Freemont, 2000; Lorick et al., 1999).
To address the question of which regions of Rep and Topors are involved in the Rep–Topors interaction, a series of pGBT9–Rep constructs containing various parts of the Rep ORF fused to the Gal4 DNA binding domain and a series of pGAD424–Topors constructs containing different parts of the Topors ORF fused to the Gal4 transactivation domain were generated. Interaction studies were then performed in yeast SFY526 cells with a quantitative liquid culture assay using CPRG as a substrate (Clontech).

The Rep sequences required for interaction were analysed after cotransformation with the pGAD424–Topors(854–1045) construct comprising the 192 C-terminal Topors amino acids. Rep M172/530 (Fig. 2A) demonstrates that Rep amino acids 1 to 171 were not absolutely necessary for the Rep–Topors interaction. Further deletion of 53 amino acids in Rep40, however, led to a complete loss of interaction (Fig. 2A). The reduced reporter gene activity observed for M1/369 and M1/243 (Fig. 2A) implies an involvement of amino acids in the central part of the Rep coding region. However, a reduced expression level or improper folding of the corresponding fusion proteins cannot be excluded. A point mutation in the Rep nucleotide binding site changing lysine 340 to histidine, which abolishes the ATPase and helicase activities of the large Rep proteins, did not abolish binding to the C terminus of Topors (Fig. 2A, Rep68K340H).

p53, which also binds to Topors, shares some properties with the Rep proteins, such as transcriptional repression of unrelated promoters (Ko & Prives, 1996; Murphy et al., 1996). For this reason the Topors sequences required for interaction with p53 were analysed in parallel with those required for interaction with Rep. In these experiments construct pGBT9–M1/481, containing Rep amino acids 1 to 481, or plasmid pVA3 (Iwabuchi et al., 1993), containing murine p53 amino acids 72 to 390, was used for cotransformations, respectively. The 192 C-terminal Topors amino acids were sufficient for Rep binding (Fig. 2B, Topors(854–1045)). The reduced activity that was observed for Topors(1–1045) in comparison to Topors(854–1045) might be due to lower overall expression levels of the full-length fusion protein. p53 also interacted with the Topors C terminus and, in addition, showed an interaction with the central part of the Topors protein (Fig. 2B, Topors(1–644) and Topors(415–737)). These central Topors sequences overlap with the region between Topors amino acids 456 to 888, identified as a p53 binding site in the original characterization of Topors as a p53 interacting protein (Zhou et al., 1999). In the experiments of Zhou et al. (1999) the C-terminal Topors region was not analysed. Within the C terminus of Topors (Fig. 2C) a 7 amino acid element located between Topors amino acids 911 and 917 was important for binding of both Rep and p53 (Fig. 2C, compare Topors(854–917) and Topors(854–910)). Although the presence of Topors amino acids 911 to 917 alone was not sufficient for binding (Fig. 2C, Topors(904–925)), the redundancy of Topors sequences outside this 7 amino acid element sufficient for binding of Rep and p53 (Fig. 2C, compare Topors(854–917) and Topors(911–1045)) favours the hypothesis that this element could constitute the actual binding site but requires additional sequences for proper folding.

Since the RING finger motif of Topors is closely related to that of the ICP0 family of viral transactivators (Haluška et al., 1999), we examined the impact of Topors overexpression on AAV gene expression in the absence of helper virus. Together with a cloned wild-type AAV genome (pTAV2-0; Heilbronn et al., 1990), increasing amounts of a human cytomegalovirus promoter-driven Flag-tagged Topors construct (pCATCH–Topors) were cotransfected into HeLa cells. AAV gene expression was monitored as steady state mRNA level and Rep and Cap protein expression levels 24 h post-transfection. The low levels of Rep78 and Rep52 protein expressed from

Fig. 3. Overexpression of Topors stimulates AAV gene expression in the absence of helper virus infection. HeLa cells were transfected with constant amounts (2 μg) of a wild-type AAV genome (pTAV2-0) and cotransfected with increasing amounts of the pCATCH–Topors plasmid encoding a CMV-driven Flag-tagged full-length Topors protein as indicated. Total transfected DNA amounts were adjusted with the empty vector pCATCH (Georgiev et al., 1996) (kindly provided by J. Pavlovic, Institute of Medical Virology, University of Zurich, Switzerland). At 24 h post-transfection, the cells were assayed for AAV Rep (A) and Cap (B) protein expression levels by Western blot analysis with monoclonal antibodies 303.9 (Wistuba et al., 1995) and B1 (Progen), respectively; for AAV mRNA steady state levels by Northern blot analysis (C) with a 32P-labeled AAV-2 hybridization probe; and for Flag–Topors expression levels by Western blot analysis with the monoclonal anti-Flag antibody M2 (Sigma) (E). Arrows indicate the positions of AAV Rep proteins Rep78 and Rep52, Cap proteins VP1, VP2 and VP3, and AAV p5, p19 and p40 transcripts. The presence of equal amounts of RNA in Northern blot analysis for AAV mRNAs (C) was confirmed by rehybridization with a 32P-labeled β-actin hybridization probe (D). Transfections were performed at least three times with two different DNA preparations. Representative results are shown.
pTAV2-0 in the absence of helper virus were increased in parallel to increasing amounts of cotransfected pCATCH–Topors (Fig. 3A, lanes 2–5). Faint bands corresponding in size to Rep68 and Rep40 could be detected after prolonged exposures (not shown). AAV-2 capsid proteins VP1, VP2 and VP3 could only be detected after cotransfection of high amounts of pCATCH–Topors (Fig. 3B, lane 5). The reactive bands observed in Fig. 3B (lanes 1 to 4) are due to cross-reacting cellular proteins, since they were also observed in the absence of pTAV2-0 (not shown). A concomitant increase in the steady state level of p5, p19 and p40 transcripts was also found at high concentrations of pCATCH–Topors (Fig. 3C, lanes 4 and 5), suggesting that the stimulatory effect of Topors overexpression on AAV protein levels takes place at a level preceding translation, such as transcription or RNA stability. Expression of Flag-tagged Topors protein was assayed with an anti-Flag antibody and could consistently only be detected at the highest concentration of cotransfected pCATCH–Topors plasmid (Fig. 3E), which may be due to a limiting sensitivity of the anti-Flag antibody. Neither AAV DNA replication nor the formation of infectious AAV particles was detected upon overexpression of Topors in the absence of helper virus (not shown).

In summary, the results suggest that Topors is involved in regulation of AAV gene expression in the absence of helper virus. Interaction of Rep with Topors would then provide a means for Rep-mediated regulation of AAV gene expression. Due to the presence of five PEST sequences, Topors is probably a rather short-lived protein. The domains necessary for the Rep–Topors interaction correspond to those that are needed for Rep-mediated inhibition of cellular transformation by E1A/ras (Khleif et al., 1991; Yang et al., 1992). This suggests a possible role for the Rep–Topors interaction in Rep-mediated cellular transformation, which is further emphasized by our finding that p53 can interact with the same C-terminal Topors sequences as the Rep protein. However, more information on the cellular function of Topors will be required to understand the role of the interaction of Rep with this cellular protein.

References


of cellular transformation by the adeno-associated virus rep gene. 


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