The EMBO Journal Vol.18 No.1 pp.212–228, 1999

Integration of a growth-suppressing BTB/POZ domain protein with the DP component of the E2F transcription factor

Susana de la Luna[1], K.Elizabeth Allen, Sarah L.Mason and Nicholas B.La Thangue[2]

Division of Biochemistry and Molecular Biology, Davidson Building, University of Glasgow, Glasgow G12 8QQ, UK

[1]Present address: Centre de Genètica Mèdica Molecular, Hospital Duran i Reynals, Barcelona, Catalonia, Spain

[2]Corresponding author
e-mail: N.LaThangue@bio.gla.ac.uk

Transcription factor E2F plays an important role in orchestrating early cell cycle progression through its ability to co-ordinate and integrate the cell cycle with the transcription apparatus. Physiological E2F arises when members of two distinct families of proteins interact as E2F–DP heterodimers, in which the E2F component mediates transcriptional activation and the physical interaction with pocket proteins, such as the tumour suppressor protein pRb. In contrast, a discrete role for the DP subunit has not been defined. We report the identification and characterization of DIP, a novel mammalian protein that can interact with the DP component of E2F. DIP was found to contain a BTB/POZ domain and shows significant identity with the *Drosophila melanogaster germ cell-less* gene product. In mammalian cells, DIP is distributed in a speckled pattern at the nuclear envelope region, and can direct certain DP subunits and the associated heterodimeric E2F partner into a similar pattern. DIP-dependent growth arrest is modulated by the expression of DP proteins, and mutant derivatives of DIP that are compromised in cell cycle arrest exhibit reduced binding to the DP subunit. Our study defines a new pathway of growth control that is integrated with the E2F pathway through the DP subunit of the heterodimer.

Keywords: DP-interacting protein/growth control/transcription factor E2F

Introduction

The E2F family of transcription factors plays a critical role in orchestrating early cell cycle progression. In concert with their afferent regulators, which include the retinoblastoma tumour suppressor protein pRb and G_1 cyclin-dependent kinases (cdks), E2F integrates and co-ordinates early cell cycle events with the transcription of genes required for entry into S phase (Nevins, 1992; La Thangue, 1994; Lam and La Thangue, 1994). Significantly, the pathway responsible for regulating E2F is aberrant in most human tumour cells. For example, the Rb gene frequently suffers inactivating mutations (Weinberg, 1995), and the cyclin D family, critical regulators of pRb activity, are expressed at high levels in certain tumour cells (Hunter and Pines, 1994; Hall and Peters, 1996), events that underscore the importance of E2F in growth control.

It is known that physiological E2F arises when a member of two families of proteins, E2F and DP, interact as E2F–DP heterodimers (La Thangue, 1994; Lam and La Thangue, 1994). Each E2F component harbours a *trans* activation domain in the C-terminal region which also physically associates with an appropriate pocket protein, an interaction that impedes transcriptional activation (Flemington *et al.*, 1993; Helin *et al.*, 1993; Zamanian and La Thangue, 1993). For example, E2F-1 is regulated principally by pRb (Flemington *et al.*, 1993; Helin *et al.*, 1993), and E2F-4 by p107 and p130 (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Sardet *et al.*, 1995; Vairo *et al.*, 1995). Other levels of control that influence E2F include phosphorylation (Dynlacht *et al.*, 1994; Fagan *et al.*, 1994; Xu *et al.*, 1994; Krek *et al.*, 1995; Altiok *et al.*, 1997; Dynlacht *et al.*, 1997), protein stabilization (Hateboer *et al.*, 1996; Hofmann *et al.*, 1996; Campanero and Flemington, 1997) and regulated intracellular location (de la Luna *et al.*, 1996; Magae *et al.*, 1996; Allen *et al.*, 1997; Lindeman *et al.*, 1997; Muller *et al.*, 1997), thus providing additional mechanisms that govern the activity of E2F.

Each E2F protein requires a DP protein as an obligate heterodimeric partner (La Thangue, 1994; Lam and La Thangue, 1994). DP-1 is a widespread partner for E2F family members, being present in many cell types (Bandara *et al.*, 1993, 1994; Girling *et al.*, 1993; Wu *et al.*, 1995), in contrast to, for example, DP-3 which appears to be a less frequent component (Rogers *et al.*, 1996; S.de la Luna and N.B.La Thangue, unpublished data). In this respect, it is notable that murine DP-3 differs from other members of the E2F and DP families in that the DP-3 RNA undergoes extensive processing due to alternative splicing (Ormondroyd *et al.*, 1995). Processing events in the 5′-untranslated and coding region give rise to spliced variants that are restricted in both cells and tissues (Ormondroyd *et al.*, 1995). Considering the DP-3 proteins, four distinct isoforms have been identified, referred to as α, β, γ and δ (Ormondroyd *et al.*, 1995). In addition, a form equivalent to the DP-3δ isoform has been designated human DP-2 (Wu *et al.*, 1995; Zhang and Chellappan, 1995; Rogers *et al.*, 1996) and a form equivalent to murine DP-3α has been described in human cells (Zhang and Chellappan, 1996).

Previous studies on the properties of the DP-3 proteins have allowed some novel and important regulatory mechanisms in the control of E2F activity to be uncovered. For example, the α and δ isoforms share an alternatively spliced exon, which encodes a sequence of 16 amino acid residues, referred to as the E region, that is absent from the β and γ isoforms (Ormondroyd *et al.*, 1995); this

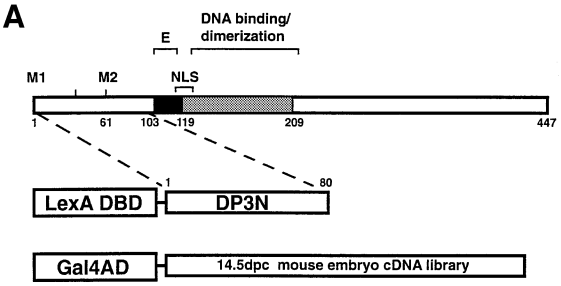
amino acid sequence is not present in the DP-1 protein (Ormondroyd *et al.*, 1995). An analysis of the role of the E region found that it functions as a nuclear localization signal (NLS), specifically in supplying one half of a bipartite NLS (de la Luna *et al*., 1996), and hence enabling the incumbent DP protein to undergo nuclear accumulation (de la Luna *et al*., 1996; Magae *et al*., 1996). Importantly, the presence of the E region, and thus nuclear accumulation, endows the DP protein with an ability to promote cell cycle progression when complexed with E2F family members, such as E2F-4 and -5, which lack an intrinsic NLS (Allen *et al*., 1997; Muller *et al*., 1997). In sharp contrast, nuclear accumulation of the E2F heterodimer mediated through pocket protein binding impedes cell cycle progression (Allen *et al.*, 1997), highlighting the importance of regulated intracellular distribution as a means towards controlling E2F activity.

Studies on the BTB/POZ domain have found that it is an evolutionarily conserved protein–protein interaction domain present in a variety of eukaryotic proteins, many of which have DNA-related functions (Bardwell and Treisman, 1994; Zollman *et al*., 1994; Albagli *et al.*, 1995). In *Drosophila melanogaster*, the BTB/POZ domain protein group is made up of transcription factors which play key roles in a variety of developmental programmes including the onset of metamorphosis, photoreceptor development, specification of abdominal segmentation and pole cell formation in the embryo, muscle recognition by nerve cells and in the development of the limb (Albagli *et al.*, 1995).

The mammalian group includes proteins involved in transcriptional regulation, with some evidence suggesting additional roles for certain BTB/POZ domain proteins in transcriptional repression (Dhordain *et al*., 1997b; Hong *et al*., 1997; David *et al*., 1998). Further, they can be important in influencing tumorigenesis as, for example, the gene encoding LAZ3/BCL6 (lymphoma-associated zinc finger 3/B cell lymphomas 6) frequently is altered by chromosomal translocation, small deletions and point mutations in non-Hodgkin lymphomas (Kerckaert *et al*., 1993; Ye *et al*., 1993). Similarly, in a subset of acute promyelocytic leukemia, *PLZF* (promyelocytic leukemia zinc finger) is fused to the *RARα* (retinoic acid receptor α) gene (Chen *et al*., 1993). Although the contribution of these mutational events to tumorigenesis remains unclear, such studies do nevertheless imply that some BTB/POZ domain proteins have an important role to play in regulating proliferation.

In this study, we report a new mammalian BTB/POZ domain protein and provide evidence that it interacts with the DP component of the E2F heterodimer. The DIP (for DP-interacting protein) protein possesses significant identity to the product of the *Drosophila* gene *germ cell-less* (Jongens *et al*., 1992). In mammalian cells, DIP is located as speckles in the nuclear envelope region and has a dominant influence on the distribution of certain DP proteins by directing them into a similar speckled pattern. DIP is capable of promoting cell cycle arrest, and DIP-dependent growth arrest is modulated by the expression of DP proteins. Our study defines a new pathway of growth control that is likely to be integrated with the E2F pathway.

A



B

Bait	Prey	Signal
Lex DBD	Gal TAD	white
Lez DBD-DP3N	Gal TAD	white
Lex DBD	Gal TAD-DIP	white
Lex DBD-DP3N	Gal TAD-DIP	blue
Lex DBD-DP3α	Gal TAD-DIP	white
Lex DBD-DP1	Gal TAD-DIP	white

Fig. 1. Yeast two-hybrid screening. (**A**) DP-3α is shown with initiating methionine (M1), the same for DP-3δ (M2), the E region (E), the nuclear localization signal (NLS) and the DNA-binding/dimerization domain. The figure shows a diagramatic representation of the bait (DP-3α amino acids 1–79 fused to the LexA DNA-binding domain) and the prey (a 14.5 d.p.c. mouse embryo cDNA library fused to the Gal4 activation domain) used in the screening. (**B**) Summary of the results from the interaction studies performed in yeast of the indicated baits and preys.

Results

Isolation of a novel DP-interacting protein

With a view towards understanding the role of the DP subunit of the E2F heterodimer, we reasoned that the N-terminal extension that occurs in the α isoform of DP-3 (Ormondroyd *et al.*, 1995) may function as a protein interaction domain. To explore this idea, we screened for proteins that interact with DP-3 in a yeast two-hybrid screen (Figure 1A). From an activation domain-tagged library prepared from 14.5 d.p.c mouse embryos, we identified two recombinants, derived from the same gene, encoding fusion proteins capable of specifically interacting with LexA DBD-DP3N (Figure 1A, and see Materials and methods). Since the cDNA sequence contained in these recombinants encoded a novel protein, we have tentatively given it the designation DIP, derived from DP-interacting protein. The interaction between LexA DBD-DP3N and DIP was specific; binding was not apparent between DIP and the LexA DNA-binding domain (Lex DBD) or DP-3N and the Gal4 activation domain (GAD; Figure 1B).

DIP, a novel member of the BTB/POZ domain family of proteins

Using the DIP cDNA, together with combined RACE and cDNA library screening (see Materials and methods), we

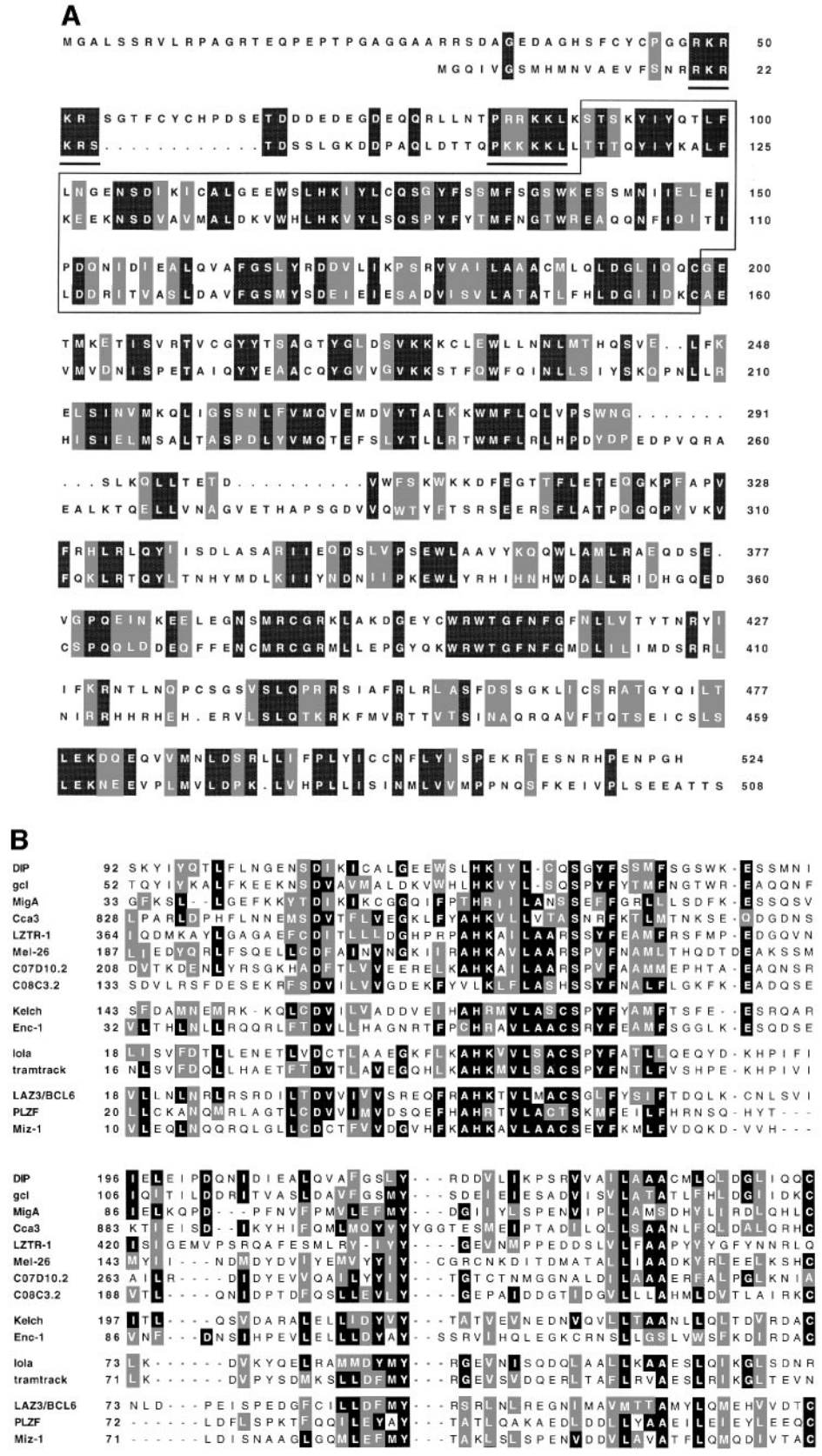


Fig. 2. DIP is likely to be the mouse homologue of the *Drosophila melanogaster* GCL. (**A**) Amino acid sequence alignment of the murine (m) DIP sequence (upper line) and *Drosophila* GCL (lower line). Only the first 508 amino acids are shown for the GCL sequence. Dark boxes indicate identical amino acids and light boxes indicate similar amino acids. Putative NLS motifs are underlined and the BTB/POZ domain is boxed. (**B**) Multiple amino acid sequence alignment of BTB/POZ domain-containing proteins. The proteins used in the alignment and their database accession numbers are as follows: *Dictyostelium discoideum* MigA, U86962; *Caenorhabditis elegans* Mel-26, U67737; C07D10.2, U13072; C08C3.2, P34324; *D.melanogaster* gcl, Q01820; kelch, A45773; lola, P42284; tramtrack, S10881; *Rattus norvegicus* Cca3, AB000216; *Mus musculus* Enc-1, U65079; *Homo sapiens* LZTR-1, D38496; LAZ3/BCL6, P41182; PLZF, Q05516; Miz-1, Y09723. Dark boxes indicate identical amino acids and light boxes indicate similar amino acids which are conserved in at least eight of 15 sequences.

isolated overlapping clones that allowed us to assemble the complete sequence of murine (m) DIP (Figure 2A). Conceptual translation of the DIP DNA sequence yielded a 524 residue polypeptide with a predicted mol. wt of 60 kDa. Searching the available DNA and protein sequence databases revealed one other protein with significant similarity to mDIP. Thus, the product of the *D.melanogaster germ cell-less* gene, GCL, which is required for proper germ cell fate specification during embryogenesis (Jongens *et al*., 1992, 1994), had 36% identity and 56% similarity with mDIP (Figure 2A). The similarity is distributed throughout both proteins, suggesting that mDIP is a close mammalian homologue of *Drosophila* GCL. Furthermore, searching the available database of expressed sequence tags has identified several human sequences with >90% homology to mDIP at the amino acid level, providing further evidence that DIP is highly conserved within mammals.

A notable feature of DIP is the presence, in the N-terminal half of the protein, of a domain previously referred to as BTB/POZ (Figure 2A and B). The BTB/POZ domain is an ~120 residue hydrophobic-rich domain present in a variety of regulatory proteins (Figure 2B) that generally fall into two groups according to the sequences present in their C-terminus. One group, which includes the *Drosophila* kelch (Xue and Cooley, 1993), mammalian calicin (von Bulow *et al*., 1995) and several poxvirus proteins (Senkevich *et al*., 1993), is characterized by a domain of ~50 residues that terminates at a glycine pair repeated several times, which has been shown to be involved in actin binding (Way *et al*., 1995). A second group is made up of DNA-binding proteins, most of which contain zinc fingers in the C-terminal region (Albagli *et al*., 1995). Like other BTB/POZ domain proteins, the BTB/POZ domain is located in the N-terminal region of DIP. However, none of the protein domains which define the BTB/POZ protein subgroups, namely actin-binding domain or DNA-related domains, are apparent in the C-terminal half of DIP, and thus DIP may constitute another subgroup of BTB/POZ proteins. Other BTB/POZ domain proteins with similar characteristics that fall within this subgroup would include *Drosophila* GCL (Jongens *et al*., 1992), human LZTR-1 (Kurahashi *et al*., 1995), rat Cca3 (Hayashi *et al*., 1997), *Disctyostelum* MigA (Escalante *et al*., 1997) and some *Caenorhabditis* putative proteins (Figure 2B).

Further analysis of the DIP cDNA revealed that the mRNA has an extensive AU-rich 3′-untranslated region (UTR), of which ~1.2 kb has been sequenced (data not shown), which contains several AREs (adenylate/uridylate-rich elements), a sequence motif known to regulate the stability of many RNAs that encode proto-oncogenes, transcription factors and cytokines (Chen and Shyu, 1995). In addition, two canonical polyadenylation signals (AAUAAA) occur in the 3′-UTR separated by ~500 bases. Northern analysis of the expression pattern of DIP RNA indicated that it is present at low levels in a wide variety of murine tissues and cell lines as a transcript of ~4 kb, although in certain tissues an additional transcript was apparent at 3.5 kb (data not shown) that may arise through the alternative utilization of the two polyadenylation signals in the 3′-UTR. In support of this idea, cDNAs with poly(A) tails at both positions were isolated during the library screen (data not shown).

DIP is a nuclear protein concentrated in the nuclear envelope region in which the BTB/POZ domain is necessary for nuclear accumulation

To assess the intracellular distribution, we expressed wild-type DIP in mammalian cells and thereafter immunostained. Wild-type DIP accumulated in a striking nuclear pattern that was characterized by discrete speckles and, by confocal microscopy, was located in the nuclear periphery in the region of the nuclear envelope (Figure 3A). This pattern was apparent when using different fixation procedures (including formaldehyde, paraformaldehyde or methanol), suggesting that its appearance was not influenced by sample preparation. The speckled distribution of DIP observed here is in general in agreement with other reports on the location of nuclear BTB/POZ proteins (Bardwell and Treisman, 1994; Dong *et al*., 1996; Dhordain *et al*., 1997a), although GCL is the only BTB/POZ domain protein ascribed to a location in the nuclear periphery (Jongens *et al*., 1994).

To gain information on the role of the domains in DIP that influence the intracellular distribution, we explored the properties of several derivatives. In the first, DIPΔ46, the staining pattern was very similar to that of wild-type DIP (Figure 3B). In the second, DIP-POZ, the entire BTB/POZ domain was retained in the N-terminal region and efficient nuclear accumulation was apparent, although, in contrast to wild-type, its nuclear distribution was uniform and lacked the discrete speckled appearance characteristic of the wild-type protein [Figure 3B, compare (i) with (ii)]. In this respect, it is noteworthy that the N-terminal region in DIP-POZ contains two candidate SV40 large T antigen-like NLSs (Kalderon *et al*., 1984) which may be responsible for the nuclear accumulation (see Figure 2A). The third DIP derivative analysed, DIPΔPOZ, which contains the C-terminal half of the protein from residue 231 to 524, failed to undergo nuclear accumulation and in many cells retained a cytoplasmic location (Figure 3Biii). Overall, therefore, these results indicate that DIP is located in the region of the nuclear envelope and further that distinct domains contribute to its intracellular distribution.

DIP contains a dimerization domain

Studies on several other BTB/POZ domain proteins have established that the domain functions in protein–protein interactions, allowing the formation of both homodimers and heterodimers either with other BTB/POZ domain proteins (Bardwell and Treisman, 1994), or with unrelated proteins (Li *et al*., 1997). We were interested in testing the possibility that DIP was capable of dimerization, and for that two versions of DIP were synthesized *in vitro*; one containing an HA tag, HA-DIP (Figure 4A, track 1), and another lacking the HA tag, DIPΔ46 (Figure 4A, track 2). Both proteins were immunoprecipitated efficiently by an antibody which recognizes a C-terminal epitope in DIP (Figure 4A, tracks 4 and 5). However, when an anti-HA antibody was used in the assay, DIPΔ46 was immunoprecipitated in an HA-DIP-dependent fashion (Figure 4A, compare tracks 6 and 7), suggesting that both proteins can form a physical complex.

The interaction domain in DIP was explored further

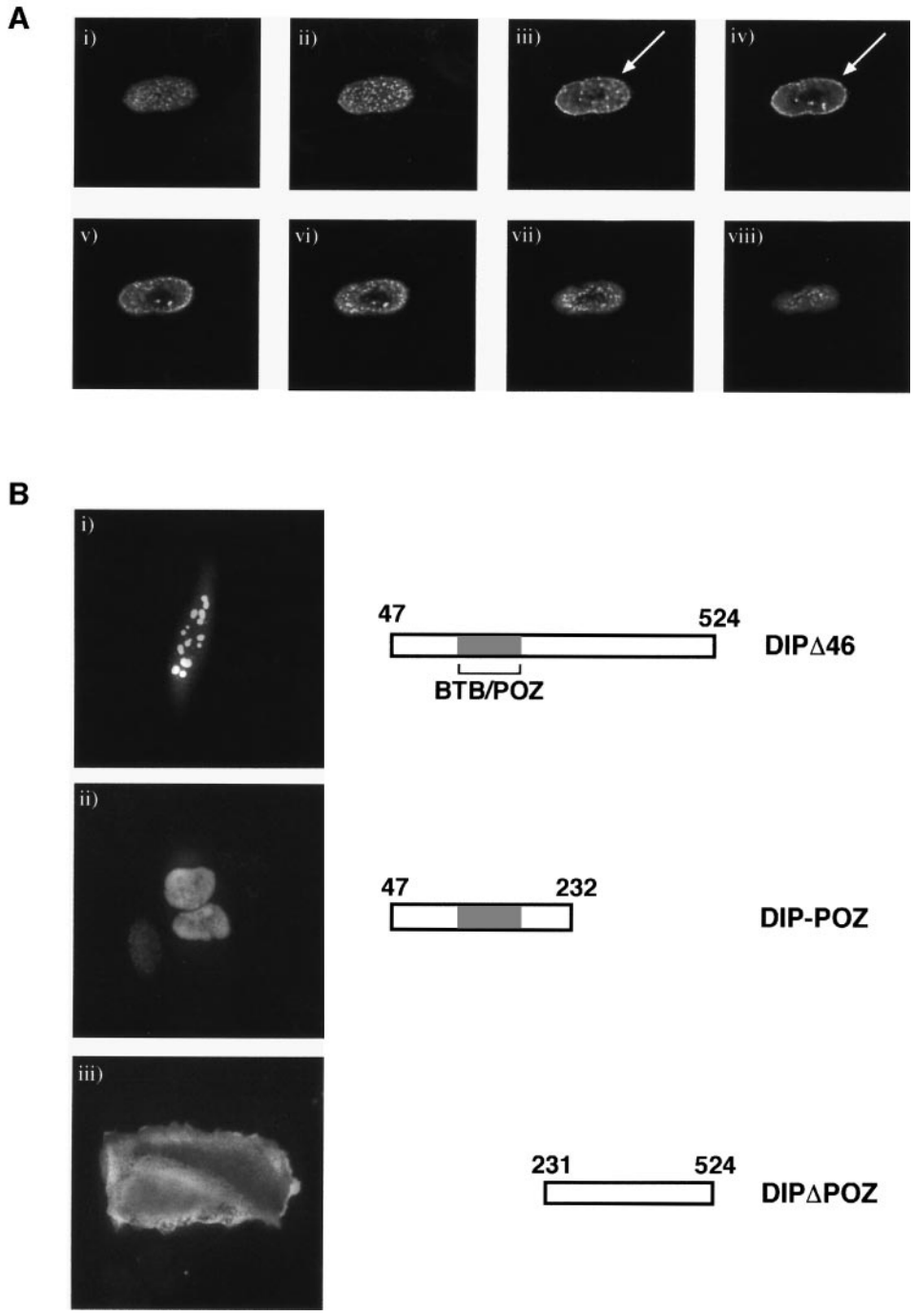


Fig. 3. Intracellular distribution of DIP protein. (**A**) U2OS cells were transfected with 3 μg of pCMV-DIP (encoding wild-type DIP) and the intracellular location of DIP examined by confocal microscopy after immunostaining with anti-DIP. The figure shows progressive serial sections of a nucleus, from dorsal (i) to ventral (viii), and the distribution of DIP. Note the concentration of DIP in the region of the nuclear envelope (indicated by arrows). (**B**) U2OS cells were transfected with 3 μg of pCMV-DIPΔ46 (i), pCMV-DIP-POZ (ii) and pCMV-DIPΔPOZ (iii), and the intracellular location of the expressed protein determined by immunostaining with an anti-T7 monoclonal antibody (ii and iii). For each panel, a diagrammatic representation of the expressed protein is shown. Note that the intracellular distribution of DIPΔ46 was essentially very similar to wild-type DIP (compare a and b).

using, first, the yeast two-hybrid assay and, secondly, the equivalent mammalian cell-based assay. Thus, wild-type DIP or mutant derivatives were fused to either the LexA DNA-binding domain or the Gal4 activation domain (Figure 4B). In yeast, oligomer formation between wild-type DIP was evident, and occurred upon deletion of the N-terminal region up to residue 231 and, furthermore, both bait and prey hybrid proteins containing only the C-terminal half of DIP (from residue 231) could interact efficiently (Figure 4B). We conclude, therefore, that the presence of the BTB/POZ domain is not required for DIP oligomerization. Rather, the data imply that oligomerization occurs through a domain located in the C-terminal half of DIP. This domain could be defined further as requiring the region from residue 231 to 283 as an interaction was not apparent

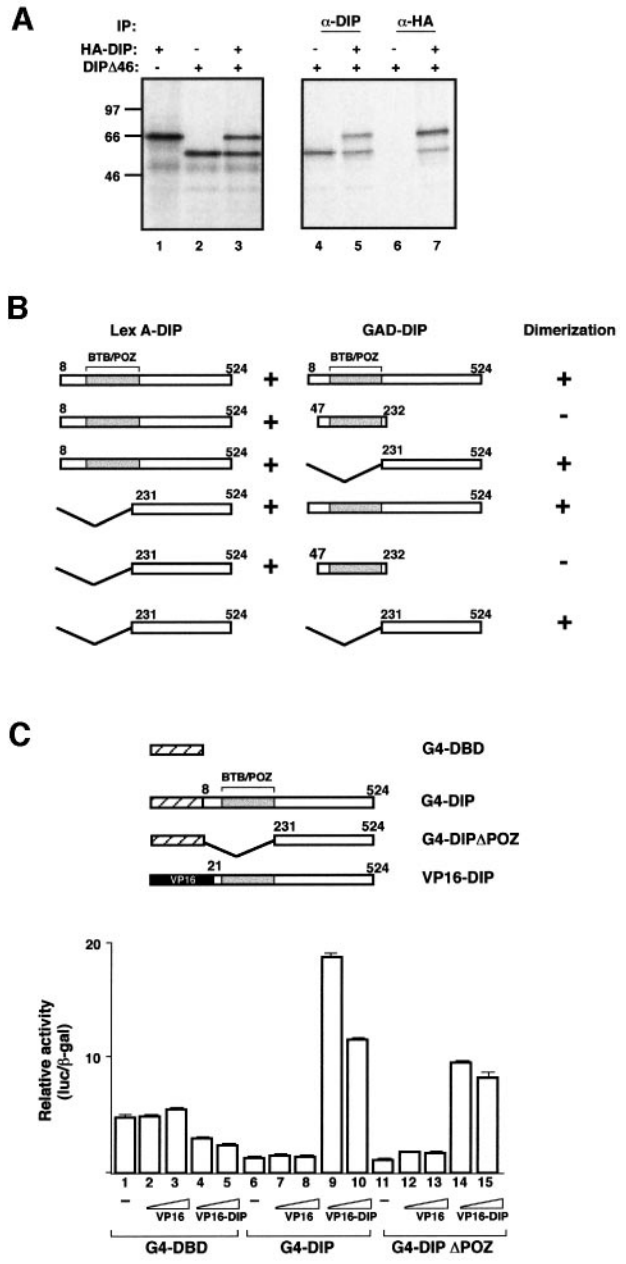


Fig. 4. DIP can homodimerize. (**A**) DIP homodimerizes *in vitro*. HA-DIP and DIPΔ46 were synthesized and ^{35}S-labelled *in vitro*, as described in Materials and methods, either alone (lanes 1 and 2) or together (lane 3). The *in vitro* products were immunoprecipitated with an anti-DIP antibody (lanes 4 and 5) or with anti-HA HA11 monoclonal antibody (lanes 6 and 7). Note that DIPΔ46 immunoprecipitated with the anti-HA monoclonal antibody only when HA-DIP was present. (**B**) DIP homodimerizes in the yeast two-hybrid system: schematic representation of the DIP hybrids used in the two-hybrid experiment, baits being fused to the LexA DNA-binding domain and preys fused to the Gal4 activation domain (GAD). For each bait, β-galactosidase background levels were determined in double transformants with Gal4-GAD alone. Values similar to background levels are given as a '–'. (**C**) The C-terminal half of DIP is responsible for DIP–DIP interaction in mammalian cells: schematic representation of the fusion proteins used. U2OS cells were transfected with pG5E1b-luc reporter plasmid (1 μg), pG4-DBD (0.5 μg; tracks 1–5), pG4-DIP (0.5 μg; tracks 6–10) or pG4-DIPΔPOZ (0.5 μg; tracks 11–15) as baits. Each bait was co-expressed with VP16-AD alone (pCMV-VP16/NLS: 1.5 μg, tracks 2, 7 and 12; 3 μg, tracks 3, 8 and 13) or DIP fused to VP16-AD (pVP16-DIP: 1.5 μg, tracks 4, 9 and 14; 3 μg, tracks 5, 10 and 15). All plates were co-transfected with pCMV-βgal (0.3 μg) as an internal control. Values are given as the ratio of luciferase to β-galactosidase activities.

when a hybrid protein DIP$^{283–524}$ was used as the prey (data not shown).

A similar conclusion was reached when DIP was studied in a mammalian two-hybrid assay where DIP was fused to the Gal4 DNA-binding domain, in G4-DIP, and the VP16 activation domain to DIP in VP16-DIP (Figure 4C). Neither VP16 nor VP16-DIP was capable of stimulating the activity of G4-DBD, although significant stimulation occurred when G4-DIP and VP16-DIP were co-expressed (Figure 4C), a result consistent with the previous data derived from the yeast two-hybrid assay. Furthermore, in the absence of the BTB/POZ domain (G4-DIPΔPOZ), an interaction was still apparent with VP16-DIP (Figure 4C). We conclude that the BTB/POZ domain is not required for DIP oligomerization but, instead, the data suggest that a C-terminal domain is required. In this respect, the BTB/POZ domain in DIP is somewhat unusual as most previously identified BTB/POZ domains function directly in facilitating dimerization.

DIP is a negative regulator of cell cycle progression

Because DIP was isolated by screening with the DP component of the E2F transcription factor, we considered that DIP may regulate cell cycle progression. To address this possibility, we assessed the effect of DIP on the cell cycle using two different assays. In the first, expression vectors for DIP were introduced into human osteosarcoma U2OS cells and its effects on the cell cycle were monitored by flow cytometry. Transfected cells were identified by introducing an expression vector for the cell surface protein CD20 and thereafter staining with an anti-CD20 monoclonal antibody, and the cell cycle kinetics of the transfected population determined by propidium iodide incorporation. The effects of DIP were studied in asynchronous and synchronous populations of U2OS cells, where synchrony had been achieved with nocodazole, a treatment that arrests cell cycle progression in mitosis.

By 36 h post-transfection of U2OS cells with either wild-type DIP or DIPΔ46, a marked increase of ~22% in the G_1 population was apparent in this particular experiment, in contrast to the control treatment with the empty vector (Figure 5A, compare cd20$^+$/mock with cd20$^+$/DIPΔ46; and data not shown); comparing a variety of data indicated that on average DIP causes an increase of between 20 and 40% in the G_1 population of transfected cells (data not shown). Similarly, when expression vectors for DIP were introduced into U2OS cells and subsequently treated with nocodazole, an increased G_1 population was still apparent compared with the empty vector-transfected cells where many cells were arrested in the mitotic fraction (Figure 5A). As a control and to confirm the effect of DIP, we assessed the CD20-negative population (taken from the cell population transfected with DIP and the control vector) after nocodazole treatment. Both populations were similar in their cell cycle profile, and resembled that for the CD20-positive population transfected with the control vector (Figure 5A, compare cd20$^-$/mock with cd20$^-$/DIP after nocodazole treatment). We conclude that exogenous DIP can promote cell cycle arrest by negatively regulating early cell cycle progression.

As a further indication of the growth-suppressing properties of DIP, we introduced DIP or DIPΔ46 into U2OS

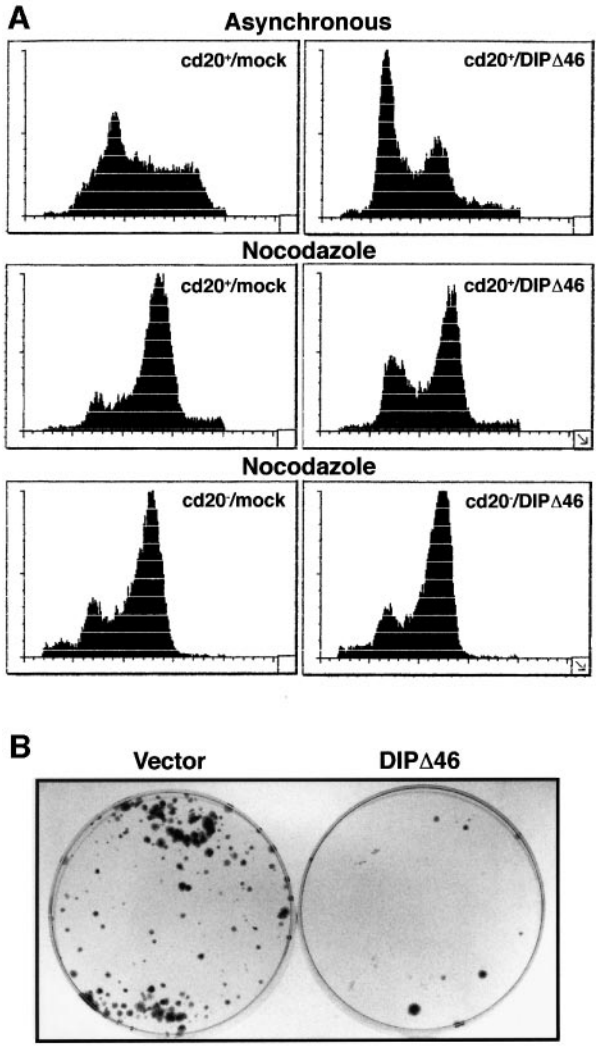


Fig. 5. DIP can cause cell cycle arrest. (**A**) U2OS cells were transfected with 16 μg of pCDNA-3 (left panels) or 16 μg of pCMV-DIPΔ46 (right panels), together with 4 μg of pCMVCD20 as described. After removal of the DNA precipitate, cells were incubated in 5% FCS and were harvested 36 h after transfection (first row). Parallel transfections were performed and treated with nocodazole 12 h before harvesting (central row). Transfected cells were identified by staining with a FITC-conjugated anti-CD20 antibody and DNA stained with propidium iodide as described in Materials and methods. For the nocodazole-treated cells, the profiles of non-transfected cells ($cd20^-$, lower row) are shown as a control for comparison with the $CD20^+$ population of transfected cells. (**B**) U2OS cells (5×10^5) plated in 6 cm dishes were transfected with 5 μg of pCDNA-3 (Vector) or 5 μg of pCMV-DIPΔ46 (DIPΔ46). The selection procedure was performed as described in Materials and methods. Stained plates representative of two different experiments are shown. Note the decrease in the number of colonies when DIP is expressed.

cells in a vector that contained an expression cassette for the neomycin resistance gene (see Materials and methods). After transfection, cells were grown under selection in the presence of G418 and the number of resistant colonies determined after 14 days in culture. As expected in the absence of DIP, colony growth was apparent (Figure 5B; Vector treatment). In contrast, far fewer colonies were evident when DIP or DIPΔ46 was expressed in the same conditions (Figure 5B; DIPΔ46 treatment), a result consistent with the flow cytometry analysis which suggested that DIP possesses the properties of a growth suppressor. The combined conclusions of these two different assays that measured the effects of DIP on proliferation strongly argue that DIP negatively regulates cell cycle progression.

DIP physically interacts with DP-3

Since DIP was isolated by screening in a yeast two-hybrid assay for proteins that could interact with DP, we assessed whether DIP could physically interact with DP proteins. However, assessing the properties of DIP in a variety of extraction procedures indicated that DIP is a highly insoluble protein. For example, in cells transfected with expression vectors encoding wild-type or a variety of DIP derivatives, the DIP proteins were usually exclusively present in the insoluble material harvested from transfected cells (Figure 6A). One mutant, ΔPOZ, could be solubilized (Figure 6A), presumably because it failed to accumulate in nuclei (Figure 3B). These properties of DIP were compared with those of endogenous p53 which, as expected, was soluble (Figure 6A). The biochemical insolubility of DIP noted here has been described previously for other BTB/POZ domain proteins (Albagli *et al*., 1995; Kim *et al*., 1998).

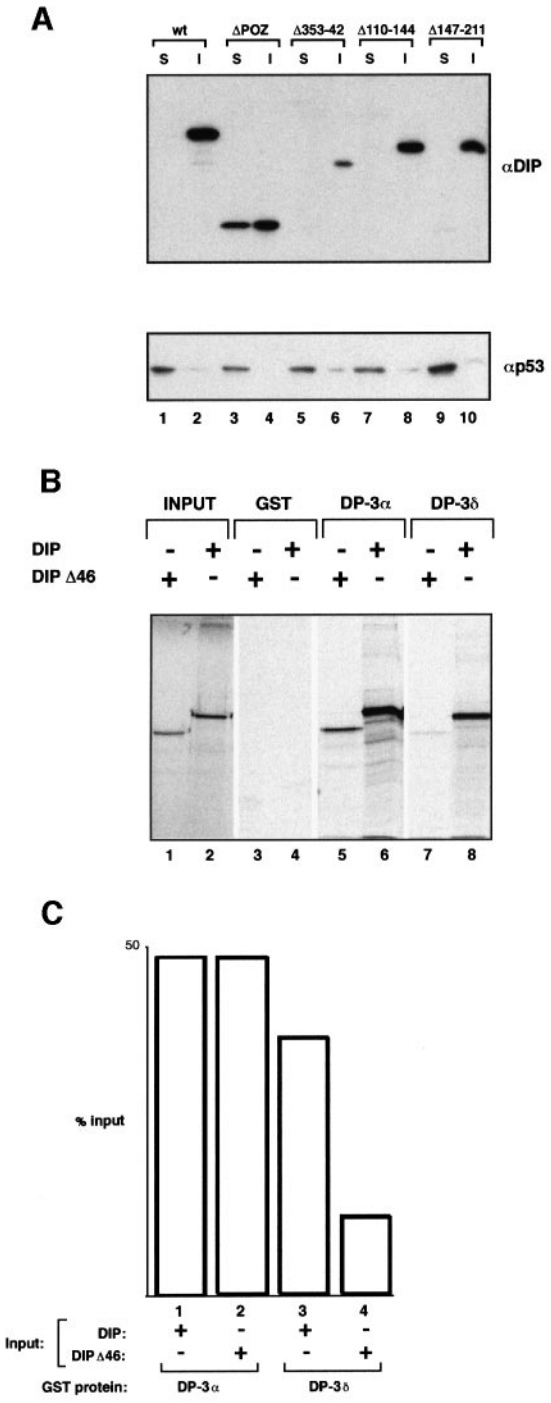
As a characterization of DIP could not involve conventional biochemical procedures, we resorted to a variety of alternative approaches. In the first, we attempted to confirm the interaction between DIP and DP-3 using GST pull-down experiments with *in vitro* translated ^{35}S-labelled DIP and bacterially expressed GST–DP-3, and thereafter measured the binding efficiency. In these experiments, DIP was retained by both GST–DP-3α and GST–DP-3δ (Figure 6B, tracks 6 and 8), although with a marginally, but reproducibly greater efficiency with DP-3α (Figure 6C, tracks 1 and 3). However, when the DIP mutant, DIPΔ46, which lacks 46 amino acid residues from the N-terminus, was used in the assay, the difference in binding efficiency became much more significant (Figure 6B, compare tracks 5 and 7; and C, tracks 2 and 4). As a control, GST alone was unable to bind to the *in vitro* translated DIP proteins (Figure 6B, tracks 3 and 4). We conclude, therefore, that DIP is able to interact with DP-3α and δ isoforms, and that an N-terminal domain in DIP is likely to be involved in discriminating between these two different DP-3 isoforms.

Next, we tested if DP-3 and DIP could associate in mammalian cells. To address this point, we performed two-hybrid assays in mammalian cells with an activation domain-tagged DIP, VP16-DIP (Figure 7A). When VP16-DIP was assayed on two different DP baits that contained either the N-terminal 79 amino acid residues of DP-3α, G4-DP3N, or the equivalent region taken from DP-1, G4-DP1N, significant stimulation of activity was apparent with G4-DP3N and not with G4-DP1N (Figure 7A, compare tracks 9 and 10 with 14 and 15). Similarly, no effect was apparent when VP16-DIP was co-expressed with the Gal4 DNA-binding domain alone (Figure 7A, tracks 4 and 5). These data suggest that DIP can associate with the N-terminal region of DP-3α in mammalian cells.

A similar assay was performed with hybrid baits in which complete DP-3α and δ sequences were fused to the Gal4 DNA-binding domain. As expected, VP16-DIP enhanced activity in the presence of G4-DP3α, an effect specific for DP-3 since similar stimulation of the DNA-binding domain of Gal4 was not evident (Figure 7B,

compare tracks 4 and 5 with 9 and 10). Furthermore, and consistent with the earlier data (Figure 6B), co-expression of VP16-DIP with G4-DP3δ also resulted in increased activity (Figure 7B, compare tracks 4 and 5 with 14 and 15). Thus, although DIP can interact with the N-terminal region of DP-3α, it is likely that an additional part of DP-3 within the sequence shared by the α and δ isoforms can also interact with DIP.

The region in DIP that is responsible for the common interaction with the α and δ isoforms was next considered. In the mammalian two-hybrid assay, a hybrid protein in which the BTB/POZ domain was fused to the VP-16 activation domain, in VP16-POZ, enhanced the activity of both G4-DP3α and G4-DP3δ (Figure 7C, compare tracks 4 and 5 with 9, 10, 14 and 15). These data suggest that the DIP BTB/POZ domain is likely to be a region responsible for the interaction with DP-3α and δ.



DIP directs DP proteins and the E2F heterodimer into the nuclear speckles

Finally, since DIP showed a characteristic staining pattern when exogenously expressed, we analysed whether co-expression of DIP with DP-3 could direct DP-3 into the DIP-dependent nuclear speckles. Such a result would be compatible with the idea of a dominant influence of DIP on DP-3, most likely through a direct interaction between both proteins. These co-localization studies were performed with DIPΔ46 because this mutant, although fully competent to bind DP-3, was able to discriminate between DP-3α and δ isoforms in the biochemical experiments described earlier (Figure 6B and C) and gave a nuclear localization pattern similar to wild-type DIP (Figure 3). Thus, the α isoform of DP-3 was co-expressed with DIPΔ46 in a variety of mammalian cell types. In U2OS cells, DP-3α efficiently accumulated in nuclei (Figure 8A), consistent with our previous results (de la Luna *et al*., 1996), to give a nuclear diffuse staining pattern. When DP-3α and DIPΔ46 were co-expressed, in striking contrast to the distribution in the absence of DIP, DP-3α became concentrated in the nuclear speckles, resulting in an almost complete coincidence between DIP and DP-3α (Figure 8, compare e and f).

We assessed the specificity of the co-localization by determining if DP-3δ, which lacks the α-specific interaction domain, was subject to a similar influence. In cells co-expressing DIPΔ46 and DP-3δ, the nuclear distribution of DIPΔ46 retained its characteristic nuclear speckles and, in the vast majority of cells, DP-3δ failed to co-localize with the DIPΔ46 speckles (Figure 8, compare g and h); very occasionally, however, DP-3δ was seen to co-localize very faintly in some of the expressing cells, a result in agreement with the weak DP3δ–DIPΔ46 interaction apparent from the *in vitro* binding data (Figure 6). These data support the idea that DP-3 is directed to the nuclear

Fig. 6. *In vitro* interactions of DIP proteins with DP-3. (**A**) Extraction properties of DIP proteins: expression vectors for wild-type DIP or the indicated mutant DIP proteins were introduced into U2OS cells, harvested and thereafter immunoblotted with an anti-DIP peptide antibody (upper) or anti-p53 (lower) as described. The fraction indicated by S contains soluble material extracted in lysis buffer, and the fraction indicated by I the insoluble material after extraction (see Materials and methods). Note that the DIP proteins (with the exception of ΔPOZ) were present in the insoluble (I) material whereas, in contrast, the majority of endogenous p53 was present in the soluble (S) fraction. (**B**) DIP and DIPΔ46 proteins were translated *in vitro* in the presence of [^{35}S]methionine (lanes 1 and 2: 10% of input). Translated proteins were mixed with 1 μg of GST protein (lanes 3 and 4), GST–DP-3α (lanes 5 and 6) or GST–DP-3δ (lanes 7 and 8) immobilized on glutathione–agarose beads, and incubated as described in Materials and methods. Bound proteins were detected by SDS–PAGE followed by autoradiography. (**C**) Summary of the amount of translated protein retained in the pull-down experiment: Tracks 1 and 2 represent the amount of DIP and DIPΔ46 proteins retained in GST–DP-3α beads, respectively; tracks 3 and 4 represent the amount of DIP and DIPΔ46 proteins retained in GST–DP-3δ beads, respectively. The amount of bound labelled protein was calculated by densitometer scanning of the autoradiographs. The values are shown as the percentage of input protein.

speckles in a DIP-dependent fashion, a process which is likely to be caused by the physical association between the two proteins.

We next examined whether the association between DIP and DP-3α was compatible with formation of the DP-3–E2F heterodimer by assessing if an associated E2F partner could likewise be directed to the DIP-dependent nuclear speckles. Consistent with our previous studies, co-expresssion of DP-3α with E2F-5, which is predominantly cytoplasmic in the absence of a nuclear targeting subunit such as DP-3α (Allen *et al*., 1997), caused the nuclear accumulation of E2F-5 (Figure 9, compare b and d). However co-expression of DIP together with DP-3α and E2F-5 caused E2F-5 to become localized in a pattern of nuclear speckles that was co-incident with the distribution of DP-3α (Figure 9, compare e and f). Therefore, DIP can direct the E2F heterodimer into the nuclear speckles.

DIP can regulate E2F site-dependent transcription

That DIP can interact with DP proteins prompted us to examine the possibility that this interaction reflected the ability of DIP to regulate E2F site-dependent transcription. To investigate this idea, we studied the effect of co-expressing DIP with either E2F-5 or E2F-1 together with DP-3 on the transcriptional activity of the cyclin E promoter, a cellular promoter that is known to be E2F responsive (Botz *et al*., 1996; Geng *et al*., 1996). In the presence of E2F-5 and either DP-3α or DP-3δ, clear activation of the cyclin E promoter was apparent (Figure 10A, tracks 5 and 6). Although the co-operation between E2F and DP components was striking, there were insignificant differences between the transcriptional effects of DP-3α and DP-3δ (Figure 10A), a result consistent with previous studies (Ormondroyd *et al*., 1995). However, the co-expression of DIP with either of the E2F-5–DP-3 heterodimers caused a considerable reduction in the level of E2F site-dependent transcription (Figure 10A, compare tracks 5 and 6 with 11 and 12). The transcriptional activity was usually diminished up to a level approaching 50%, and increased amounts of DIP failed to cause a greater reduction in activity (data not shown).

Similar results were apparent when the effects of DIP were assessed on the E2F-1–DP-3 heterodimer. In this experiment, we examined the effect of DIP on an E2F-1 mutant, E2F-1Y, which fails to bind to pRb (Helin *et al*., 1993), and therefore ruled out any indirect effects that DIP may have on the activity of pRb and its interaction with E2F-1. As observed previously (Bandara *et al*., 1993), E2F-1 could stimulate E2F site-dependent transcription in

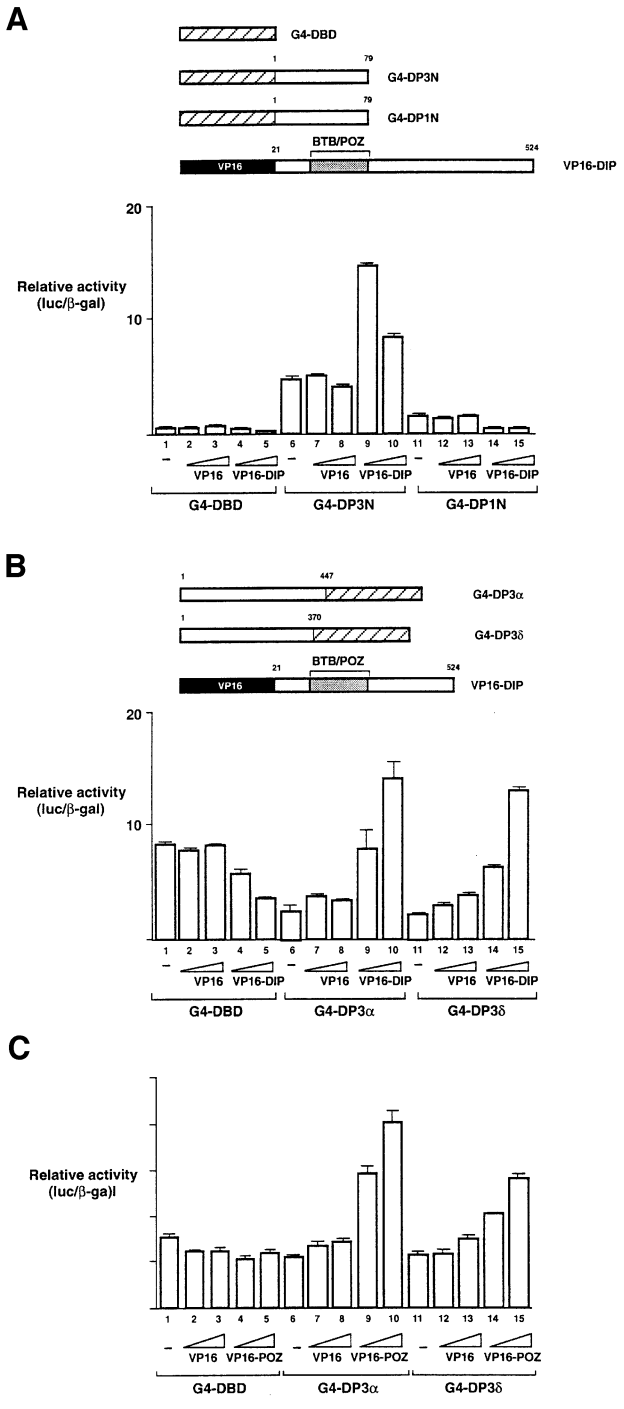


Fig. 7. DIP can interact with DP-3 isoforms. (**A**) DIP can interact specifically with the DP-3α N-terminal region: schematic representation of the fusion proteins used in the mammalian two-hybrid assay. U2OS cells were transfected with pG5E1b-luc reporter plasmid (1 μg), pG4-DBD (0.5 μg; tracks 1–5), pG4-DP3N (0.5 μg; tracks 6–10) or pG4-DP1N (0.5 μg; tracks 11–15) as baits. Each bait was co-expressed with VP16-AD alone (pCMV-VP16/NLS: 1.5 μg, tracks 2, 7 and 12; 3 μg, tracks 3, 8 and 13) or DIP fused to VP16-AD (pVP16-DIP: 1.5 μg, tracks 4, 9 and 14; 3 μg, tracks 5, 10 and 15). All plates were co-transfected with pCMV-βgal (0.3 μg) as internal control. Values are given as the ratio of luciferase to β-galactosidase activities. (**B**) DIP can interact with DP-3 isoforms: schematic representation of the fusion proteins used in the mammalian two-hybrid assay in (B) and (C). U2OS cells were transfected with pG5E1b-luc reporter plasmid (1 μg), pG4-DBD (0.5 μg; tracks 1–5), pDP3α-G4DBD (0.5 μg; tracks 6–10) or pDP3δ-G4DBD (0.5 μg; tracks 11–15) as baits. Each bait was co-expressed with VP16-AD alone (pCMV-VP16/NLS: 1.5 μg, tracks 2, 7 and 12; 3 μg, tracks 3, 8 and 13) or DIP fused to VP16-AD (pVP16-DIP: 1.5 μg, tracks 4, 9 and 14; 3 μg, tracks 5, 10 and 15). All plates were co-transfected with pCMV-βgal (0.3 μg) as internal control. Values are given as the ratio of luciferase to β-galactosidase activities. (**C**) The N-terminal half of DIP protein is responsible for the DIP–DP-3 interaction: U2OS cells were transfected with pG5E1b-luc reporter plasmid (1 μg), pG4-DBD (0.5 μg; tracks 1–5), pDP3α-G4DBD (0.5 μg; tracks 6–10) or pDP3δ-G4DBD (0.5 μg; tracks 11–15) as baits. Each bait was co-expressed with VP16-AD alone (pCMV-VP16/NLS: 1.5 μg, tracks 2, 7 and 12; 3 μg, tracks 3, 8 and 13) or $DIP^{21–232}$ fused to VP16-AD (pVP16-DIP/POZ: 1.5 μg, tracks 4, 9 and 14; 3 μg, tracks 5, 10 and 15). All plates were co-transfected with pCMV-βgal (0.3 μg) as internal control. Values are given as the ratio of luciferase to β-galactosidase activities.

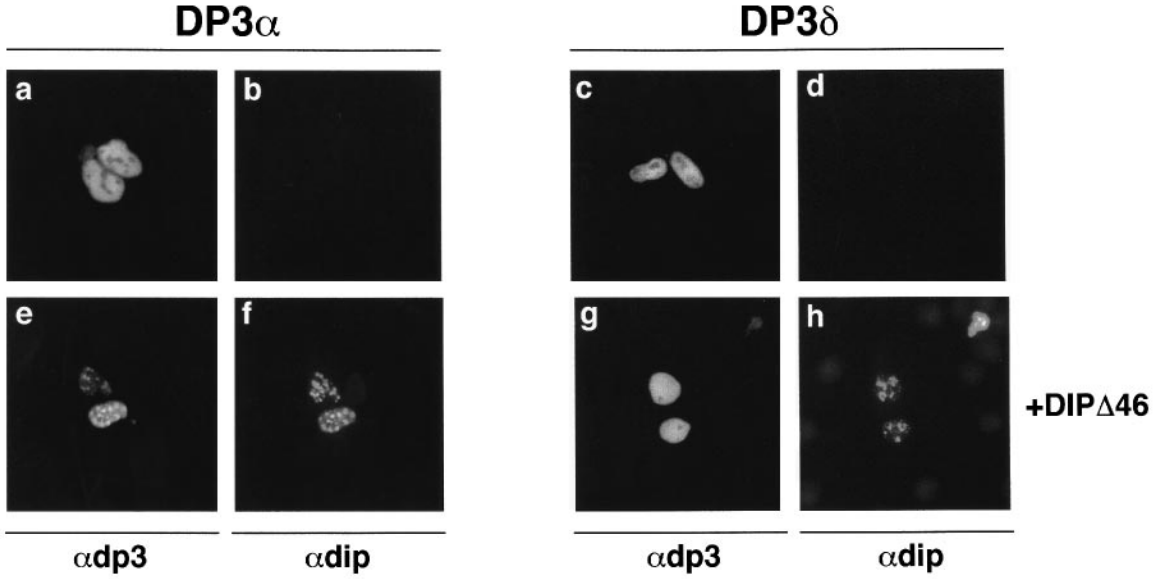


Fig. 8. DIP can translocate DP-3 into nuclear foci. U2OS cells were transfected with 3 μg of pSV-DP3α (**a**, **b**, **e** and **f**) or 3 μg of pSV-DP3δ (**c**, **d**, **g** and **h**) and pCDNA-3 (upper row) or pCMV-DIPΔ46 (bottom row). The intracellular distribution of the DP-3 isoforms and the DIP protein was determined by immunofluorescence with a rabbit polyclonal anti-DP3 antibody (a, c, e and g) and an anti-T7 monoclonal antibody (b, d, f and h). The intracellular distribution of exogenous DP-3α (a) and DP-3δ (c), exogenous DP-3α (e) and DIPΔ46 (f) in cells expressing both proteins, and of DP-3δ (g) and DIPΔ46 (h) in cells expressing both proteins is shown. Note that when DP-3 isoforms are co-expressed with DIPΔ46 only DP-3α co-localized with DIP nuclear foci.

the absence of a DP partner, although the level of activity was augmented upon co-expression of a DP partner (Figure 10B, compare tracks 2 with 5 and 6). In these conditions, the co-expression of DIP caused a reduction in the activity of the E2F-1Y–DP-3 heterodimer (Figure 10B, compare tracks 5 and 6 with 11 and 12) whilst having little apparent effect on the activity of E2F-1Y in the absence of a co-expressed DP partner (Figure 10B, compare tracks 2 with 8). Furthermore, in a similar fashion to the effect of DIP on the E2F-5 heterodimer (Figure 10A), DIP failed to cause the complete inactivation of the E2F-1–DP-3 heterodimer, but caused an ~50% reduction in the transcriptional stimulation that resulted from expression of the DP component (Figure 10B, compare track 2 with 5 and 6, and 11 and 12). Overall, therefore, these results show that DIP can diminish the activity of E2F site-dependent transcription.

DIP is a potent transcriptional repressor in mammalian cells

Based on the previous data, we wished to determine whether DIP possesses an intrinsic capacity to repress transcription. For this purpose, the ability of DIP fused to the Gal4 DNA-binding domain to affect transcription was tested on pSV-GAL-tk, a reporter construct which contains a single Gal4-binding site between the SV40 enhancer and the herpes simplex minimal thymidine kinase promoter (Figure 11A). Strikingly, G4-DIP repressed transcription from pSV-GAL-tk, an effect that was specific for DNA-bound DIP since repression was not evident either with the Gal4 DNA-binding domain alone, or when DIP was expressed without the Gal4 DNA-binding domain when a marginal increase in transcriptional activity was usually apparent (Figure 11B, compare track 3 with tracks 1 and 4). The level of repression caused by G4-DIP was similar to the effect of G4-p107 (Figure 11B, compare tracks 2 and 3). Thus, we conclude that DIP is endowed with an intrinsic ability to repress transcription in mammalian cells but, in order to do so, it needs to be targeted to the promoter context.

DP proteins modulate DIP-dependent growth arrest

The introduction of DP-3α into U20S cells caused an induction of the G_2/M phase population (Figure 12, tracks 4–6), suggesting that the α isoform could stimulate cell cycle progression. This effect was augmented by co-expressing E2F-5 with DP-3α (Figure 12, compare tracks 4–6 with 10–12), although alone E2F-5 had little effect (Figure 12, tracks 7–9).

We co-expressed the α isoform with DIP to determine if DIP could influence the effects of DP-3 on cell cycle progression or vice versa. The co-expression of DP-3α with DIP caused a marked difference in cell cycle profile. Specifically, the induction of the G_1 population by DIP was reduced markedly by co-expressing DP-3α (Figure 12, compare tracks 1–3 with 13–15) and the stimulation of cell cycle progression caused by DP-3α alone was compromised, a conclusion particularly evident from studying the size of the G_2/M population (Figure 12, compare track 6 with 15). Under these conditions, the effect of co-expressing E2F-5 was considerable. Specifically, DP-3α and E2F-5 together with DIP further reduced the size of the G_1 population and concomitantly enhanced the S and G_2/M phase population (Figure 12, compare tracks 13–15 with 16–18). Overall, these results argue that cell cycle arrest caused by DIP, namely the increased G_1 population, is modulated by co-expressing DP-3.

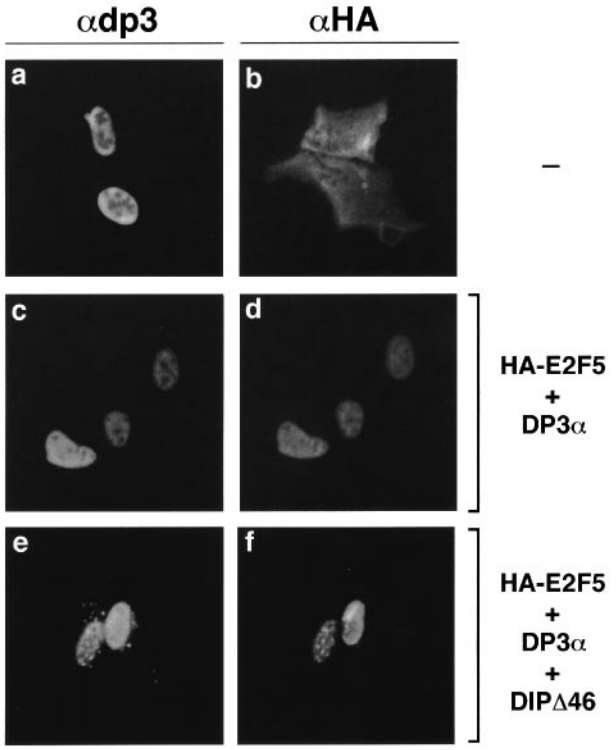


Fig. 9. DIP can translocate the E2F heterodimer into nuclear speckles. U2OS cells were transfected with 2 μg of pSV-DP3α (a, c, d, e and f) and/or 2 μg of pCMV-HAE2F5 (b, c, d, e and f) and pCDNA-3 (upper and middle row) or pCMV-DIPΔ46 (bottom row). The intracellular distribution of DP-3α and E2F-5 was determined by immunofluorescence with a rabbit polyclonal anti-DP3 antibody (a, c and e) and an anti-HA monoclonal antibody (b, d and f). The intracellular distribution of exogenous DP-3α (**a**) and E2F-5 (**b**), exogenous DP-3α (**c**) and E2F-5 (**d**) in cells expressing both proteins, and of exogenous DP-3α (**e**) and E2F-5 (**f**) in cells expressing both proteins and co-tranfected with DIPΔ46 is shown. Note that when DP-3 and E2F-5 are co-expressed with DIPΔ46 both proteins accumulated in nuclear speckles.

Multiple domains in DIP influence negative growth control and the regulation of E2F activity

We sought to gather genetic evidence that DIP can influence cell cycle progression through a modulation of E2F activity and, to pursue this question, we generated a panel of DIP mutant derivatives that were truncated at the N- or C-terminal regions, or possessed internal deletions (summarized in Figures 13A and 14A). We examined the effects of these mutants on cell cycle progression and thereafter correlated their effects with the regulation of E2F site-dependent transcription.

Since a striking effect of DIP was on the size of the G_1 population, we examined first the G_1 effect of the DIP derivatives and compared these effects with wild-type DIP. For example, DIPΔPOZ, which lacks the N-terminal half of DIP including the BTB/POZ domain (Figure 13A), was compromised in G_1 arrest and, likewise, $DIP^{46–232}$, which encompasses the BTB/POZ domain, also showed reduced cell cycle arrest (Figure 12B). An inspection of the DP-3-binding properties of ΔPOZ and $DIP^{46–232}$ indicated a significant reduction in binding relative to wild-type DIP (Figure 13C, compare lanes 1 and 2, 3 and 4, and 5 and 6). Furthermore, and consistent with this result, neither DIP derivative had a significant effect on E2F site-dependent transcription, in contrast to $DIP^{1–425}$ which behaved in a similar fashion to wild-type DIP (Figure 14D). These data suggest that the properties of DIP are influenced by distinct domains. They also support the importance of DP-3 as a target in DIP-dependent growth arrest.

We progressed on to analyse the properties of two additional mutant derivatives of DIP, namely $DIP^{\Delta 232–285}$ and $DIP^{\Delta 144–189}$ (Figure 14A). The effect of each mutant on the G_1 population was quite different, as $DIP^{\Delta 232–285}$ caused a reduced cell cycle arrest whereas, in contrast, $DIP^{\Delta 144–189}$ retained wild-type activity (Figure 14B). Furthermore, the ability to regulate cell cycle progression correlated with the DP-3-binding activity of the two DIP mutants, as $DIP^{\Delta 232–285}$ had reduced binding activity whereas $DIP^{\Delta 144–189}$ bound DP-3 as efficiently as wild-type DIP (Figure 14C, compare lanes 4, 5, 6, 7 and 9).

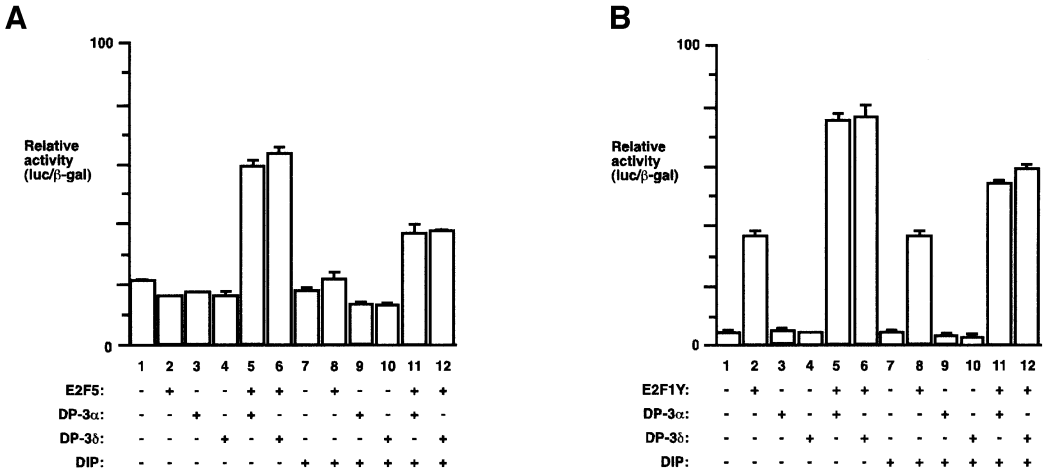


Fig. 10. DIP regulates E2F-dependent transcription. The E2F reporter pCyclinE-luc (1 μg) together with expression vectors for E2F-5 (**A**, 1.0 μg), E2F-1Y (**B**, 0.1 μg), DP-3α (2.0 μg), DP-3δ (2.0 μg) or DIP (3.0 μg) were transfected into U20S cells as indicated. The values shown represent the average of duplicate readings and represent the level of luciferase relative to the β galactosidase derived from the internal control.

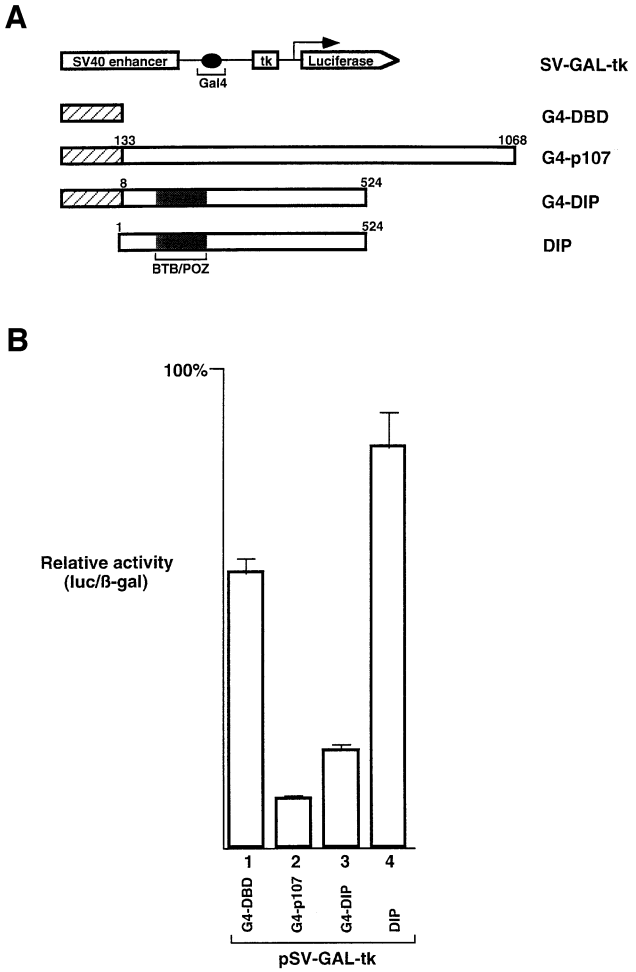


Fig. 11. DIP can repress transcription when tethered to DNA. (**A**) Schematic representation of the reporter pSV-Gal-tk and the proteins used in the assay, including a Gal4 DNA-binding domain p107 fusion protein as a positive control (Bremner *et al.*, 1995). (**B**) U2OS cells were co-transfected with 2 μg of reporter pSV-Gal-tk and 2 μg of the following plasmids: pG4-DBD (track 1), pGAL107Δ133 (track 2), pG4-DIP (track 3) and pCMV-DIPΔ46 (track 4). pCMV-βgal (0.3 μg) was included in all transfections for luciferase normalization. The value obtained with reporter alone was given an arbitrary assignment of 100.

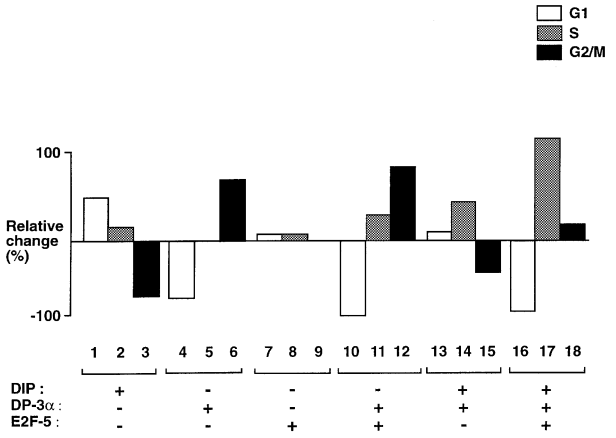


Fig. 12. Effects of DIP and DP proteins on cell cycle progression. U20S cells were transfected with expression vectors for DIP (16 μg), DP-3α (10 μg) or E2F-5 (10 μg) as indicated, together with the CD20 expression vector (4 μg). Backbone expression vectors were added to normalize the amounts of DNA in each transfection. Transfected cells were identified by staining with an FITC-conjugated anti-CD20 antiserum and DNA stained with propidium iodide as described in Materials and methods. Cells transfected with CD20 and backbone expression plasmids exhibited a cell cycle profile with ~50% cells in the G_1 phase, 20% in the S phase and 30% in the G_2/M phase of the cell cycle. The results are presented as the percentage change of cells in each phase of the cell cycle relative to CD20-expressing cells transfected with empty expression plasmids.

Finally, we investigated the properties of DIP$^{\Delta 282–285}$ on the regulation of E2F site-dependent transcription and compared its characterisitcs with those of wild-type DIP. In contrast to the inactivation of E2F-dependent transcription that resulted from co-expression of wild-type DIP, DIP$^{\Delta 232–285}$ failed to alter significantly the transcriptional activity of the cyclin E promoter driven by the DP-3–E2F-5 heterodimer (Figure 14D, compares lanes 2, 3 and 4). These data show that there is a correlation between the ability of DIP to cause cell cycle arrest, bind to DP-3 and inactivate E2F site-dependent transcription, and are consistent with the idea that DIP$^{\Delta 232–285}$ fails to affect cell cycle progression because its DP-3-binding activity is compromised.

Discussion

DIP, a new BTB/POZ domain protein

It is significant that DIP possesses a BTB/POZ domain, an interaction domain found in a growing number of eukaryotic proteins, some of which are known to be able to regulate transcription and growth (Bardwell and Treisman, 1994; Albagli *et al.*, 1995). For example, the oncoproteins LAZ3/BCL6 and PLZF, the genes for which are rearranged recurrently in hematological malignancy (Chen *et al.*, 1993; Kerckaert *et al.*, 1993), contain BTB/POZ domains together with a cluster of zinc fingers in their C-terminal domain. Three other human members have been reported as candidate tumour suppressor genes. The *HIC-1* gene is underexpressed in tumour cells due to hypermethylation (Makos Wales *et al.*, 1995). The *Miz-1* gene, cloned by means of its interaction with Myc, has a potent arrest function (Peukert *et al.*, 1997), and the *APM-1* gene is co-transcribed aberrantly with HPV68 E6 and E7 genes in a cervical carcinoma cell line (Reuter *et al.*, 1998). Other family members, such as GAGA and Mod (mdg 4), influence transcription but through mechanisms that are likely to be related to the chromatin state (Gerasimova *et al.*, 1995; Tsukiyama and Wu, 1995). Further, several BTB/POZ domain-containing transcription factors have been shown to act as transcriptional repressors (Brown *et al.*, 1991; Chang *et al.*, 1996; Dong *et al.*, 1996), and the BTB/POZ domain has been assigned an important role in mediating this repressive effect. For example, recent results have defined a functional interaction between the BTB/POZ domain proteins LAZ3/BCL6 and PLZF, and the transcriptional repressors NCoR/SMRT (Dhordain *et al.*, 1997b; Hong *et al.*, 1997; David *et al.*, 1998), thus connecting BTB/POZ-dependent repression with shared factors used by other types of transcriptional repressors.

In higher eukaryotic cells, the nuclear lamina and heterochromatin are adjacent to the inner nuclear envelope, and it is therefore possible that contact points can be established between the nuclear membrane and heterochromatin (Blobel, 1985). In this sense, HP1 (heterochromatin

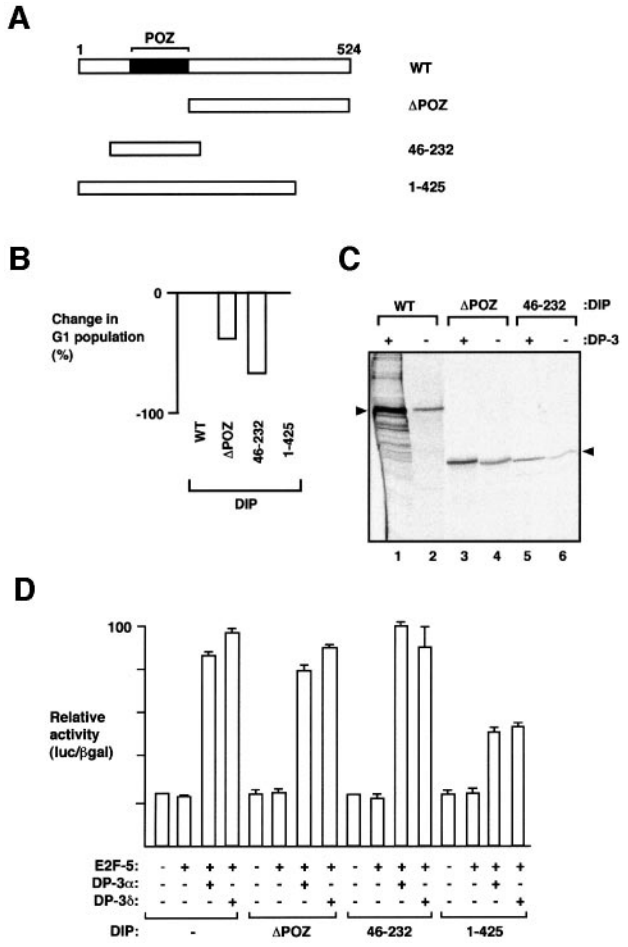


Fig. 13. Properties of the mutant DIP derivatives. (**A**) Diagrammatic summary of mutant DIP derivatives. (**B**) U20S cells were transfected with expression vectors for wild-type DIP and the indicated mutant derivatives (16 μg) as described for Figure 12. The relative change in the G_1 population of transfected cells compared with the effect of wild-type DIP is presented. (**C**) The DP-3-binding properties of the indicated mutants as assessed after *in vitro* translating wild-type DIP (lanes 1 and 2), ΔPOZ (lanes 3 and 4) and $DIP^{46–232}$ (lanes 5 and 6) in the presence of [^{35}S]methionine. Translated proteins were mixed with either 1 μg of GST–DP-3α (lanes 1, 3 and 5) or GST (lanes 2, 4 and 6) immobilized on glutathione–agarose beads, and treated as described. Note that in contrast to the binding of wild-type DIP, the ΔPOZ and $DIP^{46–232}$ proteins bind much less efficiently to DP-3. (**D**) The E2F reporter pCyclinE-luc (1 μg) together with expression vectors for E2F-5 (0.5 μg), DP-3α (0.5 μg), DP-3δ (0.5 μg) or the indicated DIP derivatives (2.0 μg) were transfected into U20S cells and assayed as described for Figure 10.

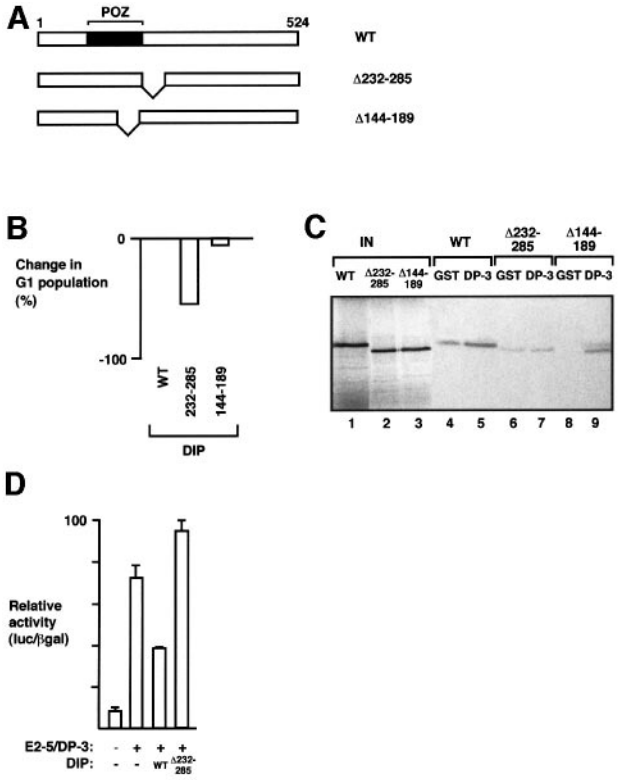


Fig. 14. Properties of a subtle DIP mutant. (**A**) Diagrammatic summary of mutant DIP derivatives. (**B**) U2OS cells were transfected with expression vectors for wild-type DIP and the indicated mutant derivatives (16 μg) as described for Figure 12. The relative change in the G_1 population of transfected cells compared with the effect of wild-type DIP is presented. (**C**) The DP-3-binding properties of the indicated DIP mutants, namely wild-type DIP (lanes 4 and 5), $DIP^{Δ232–285}$ (lanes 6 and 7) and $DIP^{Δ144–189}$ (lanes 8 and 9), was assessed as described in Figure 13; lanes 1, 2 and 3 show the input *in vitro* translated proteins. Translated proteins were mixed with either GST–DP-3 (lanes 5, 7 and 9) or GST (lanes 4, 6 and 8) and treated as described. Note that $DIP^{Δ232–285}$ has reduced binding activity whereas $DIP^{Δ144–189}$ has similar activity to wild-type DIP. (**D**) The E2F reporter pCyclin E-luc (1 μg) together with expression vectors for E2F-5 (0.5 μg), DP-3δ (0.5 μg), or the indicated DIP derivatives (2.0 μg) were transfected into U20S cells and analysed as described for Figure 10.

protein 1), which is likely to be a structural adaptor with a role in assembling macromolecular complexes in chromatin, has been shown to interact with the lamin B receptor, an integral protein of the inner nuclear envelope (Ye and Worman, 1996). Furthermore, in yeast, positioning chromatin in the perinuclear region can facilitate transcriptional silencing (Andrulis *et al*., 1998). Perhaps, given the implied role of some BTB/POZ domain proteins in chromatin control (Gerasimova *et al.*, 1995; Tsukiyama and Wu, 1995) and the established interaction between some BTB/POZ domain proteins and co-repressors which act through histone modification (Dhordain *et al.*, 1997b; Hong *et al.*, 1997; David *et al*., 1998), DIP might accumulate in nuclear compartments that house a certain type of chromatin structure.

DIP is related to Drosophila GCL

The *D.melanogaster* BTB/POZ protein GCL (Jongens *et al.*, 1992) has the greatest identity with DIP, showing an overall level of 36%. The mechanism of action of GCL is not clear, although it is known to be necessary for germ cells to complete their differentiation programme (Jongens *et al*., 1992, 1994). However, two interesting and relevant properties of *Drosophila* GCL have been described previously. First, it is known that GCL is a nuclear protein localized in the proximity of nuclear pores of the germ cell precursors (Jongens *et al*., 1994) and secondly, the overexpression of GCL in embryos causes a delay in mitosis during the pole bud nuclear divisions at the syncytial blastoderm stage (Jongens *et al.*, 1994), suggesting a role for GCL in regulating cell cycle control.

As yet, we do not know whether the conservation between GCL and DIP reflects a common function, although the high level of DIP RNA during murine spermatogenesis is consistent with a role for DIP in germ cell specification (S.de la Luna and N.B.La Thangue,

unpublished data). However, other properties of DIP, such as the low but widespread expression of DIP RNA in tumour cell lines, together with its effects on cell proliferation imply perhaps a more widespread role for DIP than previously assigned to GCL. Thus, whilst we find the relationship between DIP and GCL to be provocative and interesting, it is possible that DIP plays a more general physiological role.

DIP is integrated with the E2F pathway

It is clear from the large body of evidence that already exists that E2F plays an instrumental role in co-ordinating and integrating early cell cycle progression (La Thangue, 1994; Lam and La Thangue, 1994). E2F-binding sites, which occur in the promoters of a wide variety of genes required for proliferation, can function as either positive or negative regulators of transcription activity (Muller, 1995). Indeed, the physical association of pRb with E2F, in which pRb conceals the *trans* activation domain (Flemington *et al*., 1993; Helin *et al.*, 1993), combined with the intrinsic capacity of pRb to repress transcription (Bremner *et al.*, 1995), are features that are likely to be critical in the control of E2F activity.

Our study has identified DIP as a regulatory protein that can influence E2F activity. However, in contrast to the pRb family, where the association with E2F is direct and occurs through the C-terminal region in each E2F family member (Flemington *et al.*, 1993; Helin *et al.*, 1993), the interaction of DIP depends upon the DP family member. This view is supported by a variety of data presented in this study, including the isolation of DIP with a DP 'bait' in the two-hybrid screen, the *in vitro* physical interaction between DIP and DP proteins, the mammalian two-hybrid assays, the DP-dependent regulation of E2F activity by DIP and the DIP-directed intracellular localization of E2F. Moreover, the analysis of mutant derivatives of DIP and their interaction with DP proteins support a role for this interaction in mediating the functional effects of DIP on cell cycle progression. Overall, therefore, these results suggest that the activity of DIP is integrated with the E2F pathway.

However, a model that explains the physiological relevance of DIP in the regulation of E2F activity and cell cycle control has to encompass the results from the analysis of the mutant DIP derivatives, which although supporting the importance of the DP subunit do not rule out the possibility that multiple domains in DIP may influence its growth-regulating capacity. One possible model predicts that the growth-regulating properties of DIP are mediated through the integration of DIP activity with multiple pathways (Figure 15), and that the interplay between DIP and the E2F pathway presented in this study represents one such pathway. Such an idea is compatible with previous studies which have defined the BTB/POZ domain as an interface capable of protein–protein interactions with either other BTB/POZ domains or unrelated proteins (Bardwell and Triesman, 1994; Li *et al*., 1997), and the results from this study showing that DP proteins interact with a domain in DIP other than the BTB/POZ domain.

Nevertheless, our study has defined a new pathway of growth control that is integrated with E2F and mediated through the novel BTB/POZ domain DIP, and allows a number of significant and novel conclusions to be reached on the role of the DP subunit in the E2F heterodimer. Moreover, the negative growth-regulating properties of DIP are analogous to those possessed by pRb, the gene for which is mutated with high frequency in a variety of human malignancies. It will be interesting to establish whether the gene for DIP similarly suffers inactivating mutations in tumour cells and the influence of such events on E2F activity.

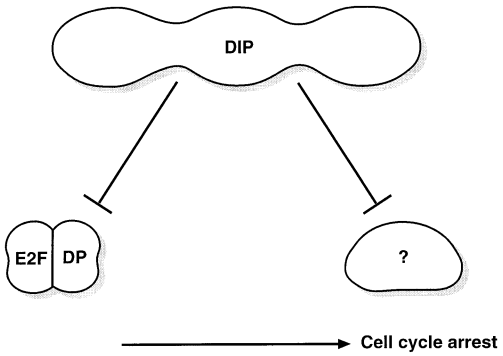


Fig. 15. Model for DIP-dependent growth control. It is envisaged that the effects of DIP on cell cycle progression are exerted through an interplay not only with the E2F pathway, mediated through an association with DP proteins, but with at least one additional as yet unidentified pathway of control.

Materials and methods

Yeast two-hybrid screening

Yeast two-hybrid screening was performed using as a bait the N-terminal 79 amino acids from mouse DP-3α fused to the DNA-binding domain of LexA in pLex(His) (Buck *et al*., 1995). The *Saccharomyces cerevisiae* strain CTY-10d, which carries an integrated *lacZ* reporter gene under the control of a LexA-responsive promoter, was transformed with the DP-3α bait and a mouse 14.5 d.p.c. embryonic cDNA library fused to the Gal4 activation domain (Chevray and Nathans, 1992), and double transformants were plated in the appropriate selective medium. About 2×10^6 double transformants were screened by filter assay for the induction of β-galactosidase according to standard procedures. Two positives were obtained, and plasmids containing the prey sequences were rescued and checked by back transformation with the bait into yeast. In order to obtain a full-length cDNA clone, an F9EC cDNA library was screened by hybridization with cDNA fragments and several clones isolated and sequenced. 5′ End sequences were obtained using RACE on mouse testis RNA (Clontech) and gene-specific oligonucleotides.

Sequences of the cDNA clones were determined in both strands manually (Sequenase, Amersham) or with an ABI dye terminator cycle sequencing-ready reaction kit (Perkin Elmer) and an automated DNA sequence analyser. Sequences were assembled into contigs with the Seqman programme from the DNAStar package (DNASTAR, Inc.). Database searches and sequence comparisons were done using the following programs provided by the National Center for Biotechnology Information: BLAST (Altschul *et al*., 1990), gapped BLAST and PSI-BLAST (Altschul *et al.*, 1997).

For analysing protein–protein interactions in yeast, CTY-10d cells were transformed with various combinations of plasmids expressing LexA DNA-binding domain (DBD)-tagged and Gal4 activation domain (GAD)-tagged molecules. Transformants were plated in the appropriate selective media and β-galactosidase activity determined for at least three independent colonies as described (Ormondroyd *et al*., 1995).

Plasmids

Yeast two-hybrid assay. pGAD, pLex(His), pLex-DP3α and pLex-DP3δ have already been described (Buck *et al*., 1995; Ormondroyd *et al.*, 1995). LexA-DBD derivatives were constructed by cloning the appropriate DNA segments from DP-3 genes and DIP cDNA into pLex(His). For generating

the Gal4-AD derivatives, plasmid pACT-II (Clontech) was used as backbone vector.

Mammalian two-hybrid assay. pG4-DBD was constructed by cloning a *Hin*dIII–*Eco*RI fragment from pSG424 (Sadowski and Ptashne, 1989) containing the Gal4 DNA-binding domain (amino acids 1–147) into pCDNA-3 (Invitrogen). pG4DP-3N and pG4DP-1N were constructed by fusing the nucleotide sequence corresponding to the first 79 amino acids of mouse DP-3 and DP-1 in-frame with the Gal4 DNA-binding domain in pG4-DBD. pG4-DIP was made by insertion of a *Sal*I–*Eco*RV fragment coding amino acids 8–524 of DIP into pG4-DBD. pDP3α-G4DBD and pDP3δ-G4DBD are C-terminal fusion proteins with the G4-DBD and were made by inserting a PCR product containing G4-DBD (amino acids 1–147) into a *Bam*HI site of pSV-DP3α and pSV-DP3δ (de la Luna *et al.*, 1996). Two DIP fragments encoding amino acids 23–524 and 21–232 were fused downstream of the VP16 activation domain in pCMV-VP16/NLS (N.Shikama and N.B.La Thangue, submitted) to generate pVP16-DIP and pVP16-POZ. Reporter vectors pG5E1b-luc and pSV-Gal-tk-luc have already been described (Lee *et al*., 1998)

Mammalian expression vectors. the following expression vectors have been already described: pSV-DP3α and pSV-DP3δ (de la Luna *et al*., 1996), pCMV-HAE2F5 (Allen *et al*., 1997), pCMV-βgal (Zamanian and La Thangue, 1993), pGal-107Δ133 (Bremner *et al*., 1995), pCyclinE-luc (Botz *et al*., 1996) and pSG5 (Green *et al.*, 1988). To construct pCMV-DIP and pCMV-HADIP, full-length DIP was cloned into pCDNA-3 and pCMV-HA1 (Lee *et al*., 1998), respectively. For generating DIP mutant tagged expression vectors, first the sequence for a T7 epitope was cloned into pCDNA-3 to produce N-terminus T7-tagged versions. The plasmids were used as a backbone vector for inserting a *Not*I–*Eco*RV fragment (amino acids 47–534), a *Not*I–*Xho*I fragment (amino acids 47–232) and an *Xho*I–*Eco*RV fragment (amino acids 231–534) from DIP full-length cDNA to generate pCMV-DIPΔ46, pCMV-DIP/POZ and pCMVDIPΔPOZ, respectively. All DIP derivative mutants were made using pCMV-DIP as the original plasmid and by cutting, filling in and ligating compatible endonuclease restriction sites within the DIP sequence to generate a continuous reading frame. DIP1-425, *Cla*I; DIP$^{\Delta 232–285}$, *Xho*I–*Pvu*II; DIP$^{\Delta 144–189}$, *Ssp*I–*Sph*I.

Transfections

Human osteosarcoma U2OS cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS). Transfections were carried out using the calcium phosphate precipitation method. Cells were plated out 24 h before transfection at 1×10^5 per 6 cm dish (two-hybrid assays, immunostaining and transcription assays) or 1×10^6 per 10 cm dish (flow cytometric analysis), washed and refed after 16 h in the presence of the DNA precipitate, and harvested and processed at a final time of 36 h post-transfection. DNA amounts were kept constant by adding pCDNA-3 or pSG5 when required. pCMV-β-galactosidase was used as an internal standard for transfections. Luciferase and β-galactosidase activities were measured in duplicate plates for each point.

For the G418 selection, cells on 6 cm dishes were transfected as described above. After washing, cells were trypsinized and plated at 1:10 dilution in 10 cm dishes. Next day, antibiotic selection was applied starting at 500 μg/ml G418 (Sigma). Cells were refed with medium with fresh antibiotic every 3–4 days until colonies were apparent (~3 weeks). Cells were washed with phosphate-buffered saline (PBS), fixed with 10% formaldehyde and stained with 1% crystal violet (w/v) in 1% formaldehyde.

To arrest cells at the G_2/M phase of the cell cycle, cells transfected as described above were treated with 40 ng/ml nocodazole in dimethylsulfoxide (DMSO) for 12 h prior to harvesting. Control cells were treated with DMSO alone.

Protein expression was checked for DIP and its derivatives by Western blotting using total cell extracts. For that, transfected cells were harvested in PBS, pelleted and resuspended in 1× SDS sample buffer. Proteins were electrophoresed in 15% SDS–polyacrylamide gels, transferred to Immobilon-P membranes (Millipore) and bands detected using ECL detection (Calbiochem). A rabbit polyclonal serum raised against a DIP-specific peptide was used at a 1:500 dilution, and peroxidase-conjugated anti-rabbit antibody (Amersham) was used as secondary antibody.

For the solubility assay, transfected cells were harvested and resuspended in lysis buffer [50 mM Tris–HCl pH 8.0, 400 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1% NP-40 and a protease inhibitor cocktail from Boehringer Manheim]. After being kept on ice for 30 min to allow solubilization, extracts were centrifuged at 5000 r.p.m. for 5 min. Supernatants from this step were considered as the soluble fraction (S fraction). The insoluble material contained in the pellets (I fraction) was resuspended in SDS sample buffer. Proteins were detected as described above using the anti-DIP polyclonal antibody and the anti-p53 monoclonal antibody DO1 (Santa Cruz).

Immunostaining

Cells grown on coverslips were washed in PBS and treated at room temperature as follows with PBS washings after each step: fixation in 4% paraformaldehyde in PBS for 15 min, permeabilization in 1% Triton X-100 in PBS for 10 min, blocking in 5% FCS in PBS for 15 min, incubation with primary antibody in 1% FCS in PBS for 30 min and incubation with secondary antibodies in 10% FCS in PBS for 30 min. Coverslips were mounted in Citifluor (Citifluor Ltd), and cells photographed with an Olympus B ×60 or confocal microscope.

The following primary antibodies were used: anti-HA monoclonal antibody HA11 (1:1000, Babco), anti-T7 tag monoclonal antibody (1:10 000, Novagen), anti-DIP polyclonal (1:200) and anti-DP3 rabbit polyclonal antibody (de la Luna *et al.*, 1996). The secondary antibodies goat fluorescein isothiocyanate (FITC)-conjugated anti-rabbit and goat tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse (Southern Biotechnology Associates, Inc.) were used at a 1:200 dilution.

Flow cytometry

DNA transfections included 4 μg of pCMVcd20 and 16 μg of the plasmid to assay. After transfection, cells were detached by treatment with cell dissociation solution (Sigma). Approximately 1×10^6 cells were incubated at 4°C with 20 μl of the FITC-conjugated anti-cd20 antibody (Becton Dickinson) for 30 min. Cells were washed twice with PBS and fixed in 50% ethanol in PBS at 4°C for at least 1 h. Fixed cells were washed and resuspended in 50 μg/ml propidium iodide containing 125 U/ml RNase A. Analysis was done on a Becton Dickinson fluorescence-activated cell sorter using the FACscan software package. About 1×10^4 events were collected for each sample.

In vitro protein interaction

The ^{35}S-labelled proteins were synthesized *in vitro* using the Promega TNT kit and T7 RNA polymerase in the presence of [^{35}S]methionine.

For immunoprecipitations, *in vitro* translated products were diluted in 200 μl of TNN buffer [50 mM Tris–HCl pH 7.4, 120 mM NaCl, 0.5% NP-40, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 20 mg/ml aprotinin] and incubated with 20 μl of a 50% slurry (w/v) of protein G–agarose beads pre-incubated with HA11 monoclonal antibody and washed in TNN. After an incubation of 1 h at 4°C, the beads were washed four times with TNN buffer and the proteins released in SDS sample buffer and detected by SDS–PAGE followed by autoradiography.

For interactions with GST proteins, *in vitro* translated products were diluted in 200 μl of incubation buffer (50 mM Tris pH 8, 100 mM NaCl, 0.5% NP-40, 1 mM DTT, 0.5 mM EDTA, 0.2 mM EGTA and 1 mM PMSF). Approximately 1 μg of GST fusion proteins or GST protein alone, purified from bacteria as described in Ormondroyd *et al*. (1995), was added in a total of 20 μl of glutathione–agarose beads and the proteins incubated at 4°C for 5 h. The beads were washed four times in incubation buffer and bound proteins detected by SDS–PAGE followed by autoradiography. Autoradiographs were quantitated by densitometry scanning using a Bio-Rad GS670 Imaging Densitometer.

Acknowledgements

We thank Marie Caldwell for help in preparing the manuscript, Kate Powell for help with the confocal microscopy, and members of our laboratory for critical comments. S.de la L. was supported by the TMR Programme of the European Union and K.E.A. by the Leukaemia Research Fund. We also thank the Medical Research Council for supporting this work.

References

Albagli,O., Dhordain,P., Deweindt,C., Lecocq,G. and Leprince,D. (1995) The BTB/POZ domain: a new protein–protein interaction motif common to DNA- and actin-binding proteins. *Cell Growth Differ.*, **6**, 1193–1198.

Allen,K.E., de la Luna,S., Kerkhoven,R.M., Bernards,R. and La Thangue,N.B. (1997) Distinct mechanisms of nuclear accumulation regulate the functional consequence of E2F transcription factors. *J. Cell Sci.*, **110**, 2819–2831.

Altiok,S., Xu,M. and Spiegelman,B.M. (1997) PPAR gamma induces

cell cycle withdrawal: inhibition of E2F/DP DNA-binding activity via down-regulation of PP2A. *Genes Dev.*, **11**, 1987–1998.

Altschul,S.F., Gish,W., Miller,W., Myers,E.W. and Lipman,D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.*, **215**, 403–410.

Altschul,S.F., Stephen,F., Madden,T.L., Schaffer,A.A., Zhang,J., Zhang,Z., Miller,W. and Lipman,D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.*, **25**, 3389–3402.

Andrulis,E.D., Neiman,A.M., Zapulla,D.C. and Stenglaz,R. (1998) Perinuclear localization of chromatin facilitates transcriptional silencing. *Nature*, **394**, 592–595.

Bandara,L.R., Buck,V.M., Zamanian,M., Johnston,L.H. and La Thangue,N.B. (1993) Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1/E2F. *EMBO J.*, **13**, 4317–4324.

Bandara,L.R., Lam,E.W.-F., Sorensen,T.S., Zamanian,M., Girling,R. and La Thangue,N.B. (1994) DP-1: a cell cycle-regulated and phosphorylated component of transcription factor DRTF1/E2F which is functionally important for recognition by pRb and the adenovirus E4 orf 6/7 protein. *EMBO J.*, **13**, 3104–3114.

Bardwell,V.J. and Treisman,R. (1994) The POZ domain: a conserved protein–protein interaction motif. *Genes Dev.*, **8**, 1664–1677.

Beijersbergen,R.L., Kerkhoven,R.M., Zhu,L., Carlee,L., Voorhoeve,P.M. and Bernards,R. (1994) E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 *in vivo*. *Genes Dev.*, **8**, 2680–2690.

Blobel,G. (1985) Gene gating: a hypothesis. *Proc. Natl Acad. Sci. USA*, **82**, 8527–8529.

Botz,J., Zerfass-Thome,K., Spitkovsky,D., Delius,H., Vogt,B., Eilers,M., Hatzigeorgiou,A. and Jansen-Durr,P. (1996) Cell cycle regulation of the murine cyclin E gene depends on an E2F binding site in the promoter. *Mol. Cell. Biol.*, **16**, 3401–3409.

Bremner,R., Cohen,B.L., Sopta,M., Hamel,P.A., Ingles,C.J., Gallie,B.L. and Phillips,R.A. (1995) Direct transcriptional repression by pRB and its reversal by specific cyclins. *Mol. Cell. Biol.*, **6**, 3256–3265.

Brown,J.L., Sonoda,S., Veda,H., Scott,M.D. and Wu,C. (1991) Repression of the *Drosophila fushi tarazu* (*ftz*) segmentation gene. *EMBO J.*, **10**, 665–674.

Buck,V., Allen,K.E., Sorensen,T., Bybee,A., Hijmans,E.M., Voorhoeve,P.M., Bernards,R. and La Thangue,N.B. (1995) Molecular and functional characterisation of E2F-5, a new member of the E2F family. *Oncogene*, **11**, 31–38.

Campanero,M.R. and Flemington,E.K. (1997) Regulation of E2F through ubiquitin–proteasome-dependent degradation: stabilization by the pRB tumor suppressor protein. *Proc. Natl Acad. Sci. USA*, **94**, 2221–2226.

Chang,C.-C., Ye,B.H., Chaganti,R.S.K. and Dalla-Favera,R. (1996) BCL-6, a POZ/zinc-finger protein, is a sequence-specific transcriptional repressor. *Proc. Natl Acad. Sci. USA*, **93**, 6947–6952.

Chen,C.-Y.A. and Shyu,A.-B. (1995) AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem. Sci.*, **20**, 465–470.

Chen,Z., Brand,N.J., Chen,A., Chen,S.J., Tong,J.H., Wang,Z.Y., Waxman,S. and Zelent,A. (1993) Fusion between a novel Kruppel-like zinc finger gene and the retinoic acid receptor-α locus due to a variant t(11,17) translocation associated with acute promyelocytic leukemia. *EMBO J.*, **12**, 1161–1167.

Chevray,P.M. and Nathans,D. (1992) Protein-interaction cloning in yeast—identification of mammalian proteins that react with the leucine zipper of jun. *Proc. Natl Acad. Sci. USA*, **89**, 5789–5793.

David,G., Alland,L., Hong,S.G., Wong,C.W., DePinho,R.A. and Dejean,A. (1998) Histone deacetylase associated with mSin3A mediates repression by the acute promyelocytic leukemia-associated PLZF protein. *Oncogene*, **16**, 2549–2556.

de la Luna,S., Burden,M.J., Lee,C.-W. and La Thangue,N.B. (1996) Nuclear accumulation of the E2F heterodimer regulated by subunit composition and alternative splicing of a nuclear localization signal. *J. Cell Sci.*, **109**, 2443–2452.

Dhordain,P. *et al*. (1997a) The BTB/POZ domain targets the LAZ3/BCL6 oncoprotein to nuclear dots and mediates homomerisation *in vivo*. *Oncogene*, **11**, 2689–2697.

Dhordain,P., Albagli,O., Lin,R.J., Ansieau,S., Quief,S., Leutz,A., Kerckaert,J.-P., Evans,R.M. and Leprince,D. (1997b) Corepressor SMRT binds the BTB/POZ repressing domain of the LAZ3/BCL6 oncoprotein. *Proc. Natl Acad. Sci. USA*, **94**, 10762–10767.

Dong,S. *et al*. (1996) Amino-terminal protein–protein interaction motif (POZ-domain) is responsible for activities of the promyelocytic leukaemia zinc finger-retinoic acid receptor-α fusion protein. *Proc. Natl Acad. Sci. USA*, **93**, 3624–3629.

Dynlacht,B.D., Flores,O., Lees,J.A. and Harlow,E. (1994) Differential regulation of E2F transactivation by cyclin cdk2 complexes. *Genes Dev.*, **8**, 1772–1786.

Dynlacht,B.D., Moberg,K., Lees,J.A., Harlow,E. and Zhu,L. (1997) Specific regulation of E2F family members by cyclin-dependent kinases. *Mol. Biol. Cell*, **17**, 3867–3875.

Escalante,R., Wessels,D., Soll,D.R. and Loomis,W.F. (1997) Chemotaxis to cAMP and slug migration in *Dictyostelium* both depend on migA, a BTB protein. *Mol. Biol. Cell*, **8**, 1763–1775.

Fagan,R., Flint,K.J. and Jones,N. (1994) Phosphorylation of E2F-1 modulates its interaction with the retinoblastoma gene-product and the adenoviral E4 19-kDa protein. *Cell*, **78**, 799–811.

Flemington,E.K., Speck,S.H. and Kaelin,W.G. (1993) E2F1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. *Proc. Natl Acad. Sci. USA*, **90**, 6914–6918.

Geng,Y., Eaton,E.N., Picon,M., Roberts,J.M., Lundberg,A.S., Gifford,A., Sardet,C. and Weinberg,R.A. (1996) Regulation of cyclin E transcription by E2Fs and the retinoblastoma protein. *Oncogene*, **12**, 1173–1180.

Gerasimova,T.I., Gdula,D.A., Gerasimov,D.V., Simonova,O. and Corces,V.G. (1995) A *Drosophila* protein that imparts directionality on a chromatin insulator is an enhancer of position-effect variegation. *Cell*, **82**, 587–597.

Ginsberg,D., Vairo,G., Chittendon,T., Xiao,Z.-X., Xu,G., Wydner,K.K., De Caprio,J.A., Lawrence,J.B. and Livingston,D.M. (1994) E2F-4, a new E2F transcription factor family member, interacts with p107 and has transforming potential. *Genes Dev.*, **8**, 2939–2952.

Girling,R., Partridge,J.F., Bandara,L.R., Burden,N., Totty,N.F., Hsuan,J.J. and La Thangue,N.B. (1993) A new component of the transcription factor DRTF1/E2F. *Nature*, **362**, 83–87.

Green,S., Issemann,I. and Sheer,E. (1988) A versatile *in vivo* and *in vitro* eukaryotic expression vector for protein engineering. *Nucleic Acids Res.*, **16**, 369.

Hall,M. and Peters,G. (1996) Genetic alterations of cyclins, cyclin-dependent kinases and cdk inhibitors in human cancer. *Adv. Cancer Res.*, **68**, 67–108.

Hateboer,G., Kerkhoven,R.M., Shvarts,A., Bernards,R. and Beijersbergen,R.L. (1996) Degradation of E2F by the ubiquitin–proteasome pathway—regulation by retinoblastoma family proteins and adenovirus transforming protein. *Genes Dev.*, **10**, 2960–2970.

Hayashi,Y., Ichinose,M., Yuasa,H., Tatematsu,M. and Ishibashi,M. (1997) Cca3, the mRNA level of which transiently decreases before initiation of DNA synthesis in regenerating rat liver cells. *FEBS Lett.*, **406**, 147–150.

Helin,K., Harlow,E. and Fattaey,A.R. (1993) Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. *Mol. Cell. Biol.*, **13**, 6501–6508.

Hofmann,F., Martelli,F., Livingston,D.M. and Wang,Z.Y. (1996) The retinoblastoma gene-product protects E2F-1 from degradation by the ubiquitin–proteasome pathway. *Genes Dev.*, **10**, 2949–2959.

Hong,S.-H., David,G., Wong,C.-W., Dejean,A. and Privalsky,M.L. (1997) SMRT corepressor interacts with PLZF and with the PML-retinoic acid receptor α (RARα) and PLZF-RARα oncoproteins associated with acute promyelocytic leukaemia. *Proc. Natl Acad. Sci. USA*, **94**, 9028–9033.

Hunter,T. and Pines,J. (1994) Cyclins and cancer. *Cell*, **79**, 573–582.

Jongens,T.A., Hay,B., Jan,L.Y. and Jan,Y.N. (1992) The *germ cell-less* gene product: a posteriorly localized component necessary for germ cell development in *Drosophila*. *Cell*, **70**, 569–584.

Jongens,T.A., Ackerman,L.D. and Swedlow,J.R. (1994) *Germ cell-less* encodes a cell type-specific nuclear pore-associated protein and functions early in the germ-cell specification pathway of *Drosophila*. *Genes Dev.*, **8**, 2123–2126.

Kalderon,D., Roberts,B.L., Richardson,W.D. and Smith,A.E. (1984) A short amino acid sequence able to specify nuclear location. *Cell*, **39**, 499–509.

Kerckaert,J.P., Deweindt,C., Tilly,H., Quief,S., Lecocq,G. and Bastard,C. (1993) *LAZ3*, a novel zinc-finger encoding gene, is disrupted by recurring chromosome-3Q27 translocations in human lymphomas. *Nature Genet.*, **5**, 66–70.

Kim,T.A., Lim,J., Ota,S., Raja,S., Rogers,R., Rivnay,B., Avraham,H. and Avraham,S. (1998) NRP/B, a novel nuclear matrix protein, associates with p110 and is involved in neuronal differentiation. *J. Cell. Biol.*, **141**, 553–566.

Krek,W., Xu,G. and Livingston,D.M. (1995) Cyclin A-kinase regulation of E2F-1 DNA binding function underlies suppression of an S phase checkpoint. *Cell*, **83**, 1149–1158.

Kurahashi,H., Akagi,K., Inazawa,J., Ohta,T., Niikawa,N., Kayatani,F., Sano,T., Okada,S. and Nishisho,I. (1995) Isolation and characterization of a novel gene deleted in DiGeorge-syndrome. *Hum. Mol. Genet.*, **4**, 541–549.

La Thangue,N.B. (1994) DRTF1/E2F: an expanding family of heterodimeric transcription factors implicated in cell-cycle control. *Trends Biochem. Sci.*, **19**, 108–114.

Lam,E.W.-F. and La Thangue,N.B. (1994) DP and E2F proteins: co-ordinating transcription with cell cycle progression. *Curr. Opin. Cell. Biol.*, **6**, 859–866.

Lee,C.-W., Sorensen,T.S., Shikama,N. and La Thangue,N.B. (1998) Functional interplay between p53 and E2F through co-activator p300. *Oncogene*, **16**, 2695–2710.

Li,S., Li,Y., Carthew,R.W. and Lai,Z.-C. (1997) Photoreceptor cell differentiation requires regulated proteolysis of the transcriptional repressor tramtrack. *Cell*, **90**, 469–478.

Lindeman,G.J., Gaubatz,S., Livingston,D.M. and Ginsberg,D. (1997) The subcellular localization of E2F-4 is cell-cycle dependent. *Proc. Natl Acad. Sci. USA*, **94**, 5095–5100.

Magae,J., Wu,C.L., Illenye,S., Harlow,E. and Heintz,N.H. (1996) Nuclear localization of DP and E2F transcription factors by heterodimeric partners and retinoblastoma protein family members. *J. Cell Sci.*, **109**, 1717–1726.

Makos Wales,M., Biel,M.A., ElDeity,W., Nelkin,B.D., Isaa,J.P., Cavenee,W.K., Keurbitz,S.J. and Baylin,S.B. (1995) p53 activates expression of HIC-1, a new candidate tumour suppressor gene on 17p13.3. *Nature Med.*, **1**, 570–577.

Muller,H., Moroni,M.C., Vigo,E., Petersen,B.O., Bartek,J. and Helin,K. (1997) Induction of S-phase entry by E2F transcription factors depends on their nuclear localization. *Mol. Biol. Cell*, **17**, 5508–5520.

Muller,R. (1995) Transcriptional regulation during the mammalian cell cycle. *Trends Genet.*, **11**, 173–178.

Nevins,J.R. (1992) E2F: a link between the Rb tumor suppresser protein and viral oncoproteins. *Science*, **258**, 424–429.

Ormondroyd,E., de la Luna,S. and La Thangue,N.B. (1995) A new member of the DP family, DP-3, with distinct protein products suggests a regulatory role for alternative splicing in the cell cycle transcription factor DRTF1/E2F. *Oncogene*, **11**, 1437–1446.

Peukert,K., Staller,P., Schneider,A., Carmichael,G., Hänel,F. and Eiulers,M. (1997) An alternative pathway for gene regulation by Myc. *EMBO J.*, **16**, 5672–5686.

Reuter,S. *et al*. (1998) *APM-1*, a novel human gene, identified by aberrant co-transcription with pappillomavirus oncogenes in a cervical carcinoma cell line, encodes a BTB-POZ-zinc finger protein with growth inhibitory activity. *EMBO J.*, **17**, 215–222.

Rogers,K.T., Higgins,P.D.R., Milla,M.M., Phillips,R.S. and Horowitz,J.M. (1996) DP-2, a heterodimeric partner of E2F—identification and characterization of DP-2 proteins expressed *in vivo*. *Proc. Natl Acad. Sci. USA*, **93**, 7594–7599.

Sadowski,I. and Ptashne,M. (1989) A vector for expressing GAL4 (1–147) fusions in mammalian cells. *Nucleic Acids Res.*, **17**, 7539.

Sardet,C., Vidal,M., Cobrinik,D., Geng,Y., Onufryk,C., Chen,A. and Weinberg,R.A. (1995) E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle. *Proc. Natl Acad. Sci. USA*, **92**, 2403–2407.

Senkevich,T.G., Muravnik,G.L., Pozdnyakov,S.G., Chizhikov,V.E., Ryazankina,O.I., Shchelkunov,S.N., Koonin,E.V. and Chernos,V.I. (1993) Nucleotide sequence of *Xho*I O fragment of ectromelia virus DNA reveals significant differences from vaccinia virus. *Virus Res.*, **30**, 73–88.

Tsukiyama,T. and Wu,C. (1995) Purification and properties of an ATP-dependent nucleosome remodeling factor. *Cell*, **83**, 1011–1020.

Vairo,G., Livingston,D.M. and Ginsberg,D. (1995) Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. *Genes Dev.*, **9**, 869–881.

von Bulow,M., Heid,H., Hess,H. and Franke,W.W. (1995) Molecular nature of calicin, a major basic protein of the mammalian sperm head cytoskeleton. *Exp. Cell Res.*, **219**, 407–413.

Way,M., Sanders,M., Garcia,C., Sakai,J. and Matsudarai,P. (1995) Sequence and domain organization of Scruin, an actin-crosslinking protein in the acrosomal process of *Limulus* sperm. *J. Cell Biol.*, **128**, 51–60.

Weinberg,R.A. (1995) The retinoblastoma protein and cell cycle control. *Cell*, **81**, 323–330.

Wu,C.L., Classon,M., Dyson,N. and Harlow,E. (1996) Expression of dominant-negative mutant DP-1 blocks cell-cycle progression in G_1. *Mol. Cell. Biol.*, **16**, 3698–3706.

Xu,M., Sheppard,K.A., Peng,C.Y., Yee,A.S. and Piwnica-Worms,H. (1994) Cyclin A–cdk2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. *Mol. Biol. Cell*, **14**, 8420–8431.

Xue,F. and Cooley,L. (1993) *kelch* encodes a component of intercellular bridges in *Drosophila* egg chambers. *Cell*, **72**, 681–693.

Ye,B.H., Lista,F., Lococo,F., Knowles,D.M., Offit,K., Chaganti,R.S.K. and Dalla-Favera,R. (1993) Alterations of a zinc-finger encoding gene, *BCL-6*, in diffuse large-cell lymphoma. *Science*, **262**, 747–750.

Ye,Q. and Worman,H.J. (1996) Interaction between an integral protein of the nuclear envelope inner membrane and human chromo-domain proteins homologous to *Drosophila* HP1. *J. Biol. Chem.*, **271**, 14653–14656.

Zamanian,M. and La Thangue,N.B. (1993) Transcriptional repression by the Rb-related protein p107. *Mol. Biol. Cell*, **4**, 389–396.

Zhang,Y.H. and Chellappan,S.P. (1995) Cloning and characterization of human DP2, a novel dimerization partner of E2F. *Oncogene*, **10**, 2085–2093.

Zhang,Y.H. and Chellappan,S.P. (1996) Transcriptional activation and expression of DP transcription factors during cell cycle and TPA-induced U937 differentiation. *Mol. Cell. Differ.*, **4**, 297–316.

Zollman,S., Godt,D., Prive,G.G., Couderc,J.L. and Laski,F.A. (1994) The BTB domain, found primarily in zinc-finger proteins, defines an evolutionarily conserved family that includes several developmentally-regulated genes in *Drosophila*. *Proc. Natl Acad. Sci. USA*, **91**, 10717–10721.

Received August 20, 1998; revised October 27, 1998; accepted October 29, 1998