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E2F-7: a distinctive E2F family member with an unusual organization of DNA-binding domains

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The E2F family of transcription factors play an important role in regulating cell cycle progression. We report here the characterization and functional properties of a new member of the human E2F family, referred to as E2F-7. E2F-7 has two separate DNA-binding domains, a feature that distinguishes E2F-7 from other mammalian E2F proteins, but resembling the organization of recently isolated E2F-like proteins from *Arabidopsis*. E2F-7 binds to DNA independently of a DP partner and delays cell cycle progression. Interestingly, E2F-7 modulates the transcription properties of other E2F proteins. A mutational analysis indicates that the integrity of both DNA-binding domains is required for cell cycle delay and transcriptional modulation. Biochemical results and protein modelling studies suggest that in binding to DNA interactions occur between the two DNA-binding domains, most probably as a homodimer, thereby mimicking the organization of an E2F/DP heterodimer. These structural and functional properties of E2F-7 imply a unique role in regulating cellular proliferation.
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Introduction

It is known that E2F transcription factors play a central role in coordinating early cell cycle progression through regulating the timely expression of genes at the G1 to S phase transition (Dyson, 1998; Stevens and La Thangue, 2003). By integrating the phosphorylation control of the retinoblastoma tumour suppressor protein (pRb) by cyclin-dependent kinase (Cdk) complexes with the transcriptional activity of E2F target genes, E2F acts to orchestrate gene activity within the cell cycle. The pathway involved in E2F control becomes aberrant in most, if not all, human tumour cells either through mutation and inactivation of the pRb gene, or through altered Cdk activity, events that lead to the inactivation of pRb and the release of E2F (Hanahan and Weinberg, 2000).

In mammalian cells E2F exists as an E2F/DP heterodimer (Stevens and La Thangue, 2003). Currently, six E2F and two DP family members have been well characterized, and consequently there is accumulating evidence, which suggests that different E2F family members perform distinct perhaps overlapping roles (Trimarchi and Lees, 2002). For example, diverse phenotypes arise in knockout mice carrying inactivated genes. Thus, *E2F-1*$^{-/-}$ mice exhibit defects in apoptosis and an increased incidence of tumours in certain tissues (Field *et al*., 1996; Yamasaki *et al*., 1996), contrasting with the inactivation of other E2F genes like E2F-4 and E2F-5, which result in markedly distinct phenotypes (Lindeman *et al*., 1998; Humbert *et al*., 2000; Rempel *et al*., 2000). This situation presumably reflects the physiologically distinct roles assigned to individual E2F subunits. Furthermore, while the concerted action of E2F-1, E2F-2 and E2F-3 promotes cell cycle progression (Wu *et al*., 2001), under certain conditions these E2F subunits can augment apoptosis (Qin *et al*., 1994; Shan and Lee, 1994). At a general level, therefore, E2F can act therefore both positively and negatively in regulating cellular proliferation.

In this respect, considerable progress has been made in understanding the mechanisms that control E2F-1-dependent apoptosis and the role of the DNA damage signalling pathway. In mammalian cells the integrity of the pathway plays a protective role by acting in checkpoint control to cause cell cycle arrest or apoptosis (Durocher and Jackson, 2001). Members of the two groups of protein kinases involved in DNA damage signalling, the sensor ATM/ATR PI-3-kinase-like-kinases and the checkpoint effector kinases, phosphorylate E2F-1 and thereby increase protein stability (Blattner *et al*., 1999; Lin *et al*., 2001; Stevens *et al*., 2003). The phosphorylation of E2F-1 by Chk2 has been directly attributed to driving the apoptotic activity of E2F-1 (Stevens *et al*., 2003), supporting the idea that E2F subunits are subject to control from different

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regulatory cues emanating from both cell cycle and apoptotic pathways.

In order to decipher the role of E2F in cell cycle control it is necessary to understand the extent and complexity of the E2F family of proteins. Towards this end, we report here the characterization and functional properties of a new member of the human E2F family, referred to as E2F-7 (De Bruin *et al*. (2003) recently reported the murine E2F-7 gene). E2F-7 has an unusual organization, possessing two separate DNA-binding domains that bind to DNA independently of DP-1, highlighting a distinguishing feature from other mammalian E2F subunits but resembling the organization of several E2F-like proteins identified in *Arabidopsis* (Kosugi and Ohashi, 2002). E2F-7 plays a role in negative growth control through the induction of cells in G1 and, most interestingly, modulates the transcription properties of other E2F proteins, like E2F-1. The integrity of both DNA-binding domains is required for these effects. Protein modelling studies predict that in binding to DNA the two DNA-binding domains can interact most probably through forming a homodimer, and thereby mimic an E2F/DP heterodimer. These structural and functional properties imply that E2F-7 performs a unique role in regulating cellular proliferation.

Results

Isolation of human E2F-7

The complete human E2F-7 sequence contains 910 amino-acid residues (Figure 1a). Mining the available protein sequence databases identified two domains that possessed significant similarity with members of the E2F family. Both domains, referred to as domains 1 and 2 (residues 160–220 and 270–375, respectively) and located in the N-terminal half of E2F-7 (Figure 1b), exhibit similarity across the conserved E2F DNA-binding domain (Figure 1c). Residues previously shown in the solved structure of E2F-4/DP-2 (Zheng *et al*., 1999) that contact the DNA bases and backbone within the E2F recognition sequence are present in domains 1 and 2, particularly the conserved RRXYD motif, present as RRIYD in domain 1 and RRLYD in domain 2 (Figure 1c). Other residues that make heterodimerization contact with the DP protein occur in domains 1 and 2 (Figure 1c). Domain 2 is larger than domain 1, and relative to domain 1 exhibits more extensive similarity with the E2F DNA-binding domain. While the homology in domains 1 and 2 firmly establishes E2F-7 as a member of the E2F family, the organization into two separate domains represents a unique feature that is not apparent in any other member of the family characterized at the present time. Other shared domains were not apparent, such as in the C-terminal transcription activation domain, and the N-terminal motif involved with cyclinA binding (Stevens and La Thangue, 2003). The E2F-7 locus is present on human chromosome 12q21 and is encoded by a 5 kb RNA expressed widely in different tissues (data not shown).

Characterization of E2F-7

The expression of ectopic E2F-7 in U2OS cells indicated that the E2F-7 polypeptide derived from pFlag-E2F-7 was about 97 kDa (Figure 2a). By immunostaining, E2F-7 was located in nuclei of transfected cells (Figure 2b). To confirm that these observations were relevant for the endogenous E2F-7 protein, we prepared several antipeptide antisera that were specific for E2F-7 (data not shown), and present results from one representative antibody. In HeLa cell extracts endogenous E2F-7 resolved as a polypeptide of similar molecular weight to the ectopic protein although, in addition, a smaller polypeptide of approximately 90 kDa was resolved (Figure 2c). Both polypeptides were competed by the E2F-7 peptide used to raise the antibody, indicating they are both related to E2F-7. The endogenous protein, in a similar fashion to the ectopic E2F-7 protein, was localized to nuclei in U2OS cells (Figure 2d). The relationship between the two endogenous E2F-7 polypeptides has yet to be resolved.

E2F-7 fails to heterodimerize with a DP partner

The two putative DNA-binding domains contain conserved residues previously shown to make contact with the DP partner in the E2F/DP heterodimer (Figure 1c). We therefore assessed if E2F-7 could interact with DP-1 both *in vitro* and in cells. In an *in vitro* protein-binding assay, while an efficient interaction was observed between E2F-1 and DP-1, we were not able to detect an interaction between E2F-7 and DP-1 (Figure 3a).

In previous studies, we used a cell-based assay to assess the formation of the E2F/DP heterodimer. Thus, exogenous DP-1 exists predominantly as a cytoplasmic protein reflecting the absence of a discrete nuclear localization signal (NLS) but becomes nuclear upon coexpression of an E2F heterodimerization partner, which possesses an NLS. In this assay, nuclear localization is imparted on DP-1 by the E2F partner as part of a heterodimer (de la Luna *et al*., 1996). As expected, when each protein was expressed alone, ectopic DP-1 was predominantly cytoplasmic and E2F-7 nuclear (Figure 3b). In cells expressing both DP-1 and E2F-7, the presence of E2F-7 failed to alter the intracellular distribution of DP-1 (Figure 3b), supporting the conclusion that E2F-7 does not bind to DP-1 in cells.

Interaction between E2F-7 DNA-binding domains

We tested whether E2F-7 could undergo homodimer formation in cells by expressing two forms of wild-type E2F-7 carrying either an HA or Flag epitope tag. By immunoprecipitation followed by immunoblotting with either pair of antibodies, both HA- and -Flag-tagged E2F-7 were present in the immunocomplex (Figure 4a),

a

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1    MEVNCLTLKDLISPRQPRLDFAVEDGENAQKENIFVDRSRMAPKTPIKNE
51   PIDLSKQKKFTPERNPITPVKLVDRQQAEPWTPTANLKMLISAASPDIRD
101  REKKKGLFRPIENKDDAFTDSLQLDVVGDSAVDEFEKQRPSRKQKSLGLL
151  CQKFLARYPSYPLSTEKTTISLDEVAVSLGVERRRIYDIVNVLESLHLVS
201  RVAKNQYGWHGRHSLPKTLRNLQRLGEEQKYEEQMAYLQQKELDLIDYKF
251  GERKKDGDPDSQEQQLLDFSEPDCPSSSANSRKDKSLRIMSQKFVMLFLV
301  SKTKIVTLDVAAKILIEESQDAPDHSKFKTKVRRLYDIANVLTSLALIKK
351  VHVTEERGRKPAFKWIGPVDFSSSDEELVDVSASVLPELKRETYGQIQVC
401  AKQKLARHGSFNTVQASERIQRKVNSEPSSPYREEQGSGGYSLEIGSLAA
451  VYRQKIEDNSQGKAFASKRVVPPSSSLDPVAPFPVLSVDPEYCVNPLAHP
501  VFSVAQTDLQAFSMQNGLNGQVDVSLASAASAVESLKPALLAGQPLVYVP
551  SASLFMLYGSLQEGPASGSGSERDDRSSEAPATVELSSAPSAQKRLCEER
601  KPQEEDEPATKRQSREYEDGPLSLVMPKKPSDSTDLASPKTMGNRASIPL
651  KDIHVNGQLPAAEEISGKATANSLVSSEWGNPSRNTDVEKPSKENESTKE
701  PSLLQYLCVQSPAGLNGFNVLLSGSQTPPTVGPSSGQLPSFSVPCMVLPS
751  PPLGPFPVLYSPAMPGPVSSTLGALPNTGPVNFSLPGLGSIAQLLVGPTA
801  VVNPKSSTLPSADPQLQSQPSLNLSPVMSRSHSVVQQPESPVYVGHPVSV
851  VKLHQSPVPVTPKSIQRTHRETFFKTPGSLGDPVLKRRERNQSRNTSSAQ
901  RRLEIPSGGAD
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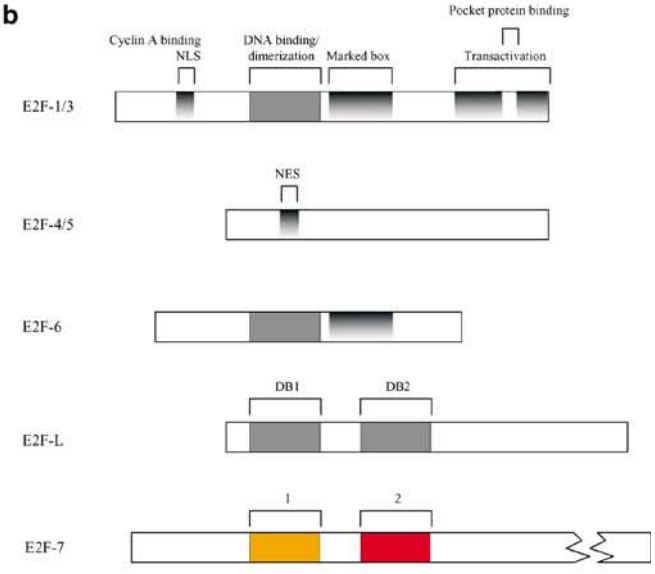
b



c

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E2F1          ---HSADGVVDLNWAAEVLKVQ-KRRIYDITNVLEGIQLIAK-----  182
E2F2          ---RSEDGVLDLNWAAEVLDVQ-KRRIYDITNVLEGIQLIRK-----  184
E2F3          ---QSPDGVLDLNKAAEVLKVQ-KRRIYDITNVLEGIHLIKK-----  233
E2F4          ---EAKDGVLDLKLAADTLAVRQKRRIYDITNVLEGIGLIEK-----   73
E2F5          ---EAKDGVLDLKAAADTLAVRQKRRIYDITNVLEGIDLIEK-----  106
E2F6          ---SAPGGILDLNKVATKLGVR-KRRVYDITNVLDGIDLVEK-----  119
E2F7 Domain1  YPLSTEKTTISLDEVAVSLGVE-RRRIYDIVNVLESLHLVSR-----  202
E2L1          ------KALLNESQD-MNMRKK-VRRLYDIANVFSSMKLIEKTHVPE  207

E2F1          KSKNHIQWLGS----HTTVG      198
E2F2          KAKNNIQWVGR----GMFED      200
E2F3          KSKNNVQWMGC----SLSED      249
E2F4          KSKNSIQWKGVG---PGCNT       90
E2F5          KSKNSIQWKGVG---AGCNT      123
E2F6          KSKNHIRWIGS----DLS-N      133
E2F7 Domain1  VAKNQYGWHGRHSLPKTLRN      222
E2L1          TKKPAYRWLGSKT             220

E2F1          SRYETSLNLTTKRFLELLSHSADGVVDLNWAAEVLKVQ--------------  162
E2F2          TRYDTSLGLLTKKFIYLLSESEDGVLDLNWAAEVLDVQ--------------  164
E2F3          TRYDTSLGLLTKKFIQLLSQSPDGVLDLNKAAEVLKVQ--------------  213
E2F4          SKHEKSLGLLTTKFVSLLQEAKDGVLDLKLAADTLAVRQ-------------   53
E2F5          SKHEKSLGLLTTKFVSLLQEAKDGVLDLKAAADTLAVRQ-------------   86
E2F6          PRFDVSLVYLTRKFMDLVRSAPGGILDLNKVATKLGVR--------------   98
E2F7 Domain2  SRKDKSLRIMSQKFVMLFLVSKTKIVTLDVAAKILIEESQDAPDHSKFKTKV  232
E2L1          ---ERTLWLLAQNFVKLFLCSDDDLVTFDSATKALLNESQD----MNMRKKV  184

E2F1          KRRIYDITNVLEGIQLIAKKSKNHIQWL    191
E2F2          KRRIYDITNVLEGIQLIRKKAKNNIQWV    193
E2F3          KRRIYDITNVLEGIHLIKKKSKNNVQWM    242
E2F4          KRRIYDITNVLEGIGLIEKKSKNSIQWK     82
E2F5          KRRIYDITNVLEGIDLIEKKSKNSIQWK    115
E2F6          KRRVYDITNVLDGIDLVEKKSKNHIRWI    127
E2F7 Domain2  -RRLYDIANNVLT-LAL-IKKVHVTSSDE    358
E2L1          -RRLYDIANVFSS-MKL-IEKTHVPETKK   210
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arguing that E2F-7 molecules can interact. While these results do not preclude an intramolecular interaction between domains 1 and 2 within E2F-7, they do support the idea of intermolecular interactions between different E2F-7 molecules.

To gain further support for this idea, we prepared a panel of E2F-7 derivatives containing either domain 1 or 2, including one in which the conserved DNA-binding motif RRLYD (residues 332–336) in domain 2 had been removed (Figure 4b). By measuring the ability of each E2F-7 derivative to bind to E2F-7 *in vitro*, we found that wild-type E2F-7 could bind to domain 1, and further that domain 1 could interact with either domain 1 or 2 (Figure 4c). Furthermore, the domain 2 mutant failed to bind to domain 1 (Figure 4c). Combined with the lack of binding to DP-1, these results suggest that domains 1 and 2 can interact with each other.

Modelling the structure of the E2F-7 DNA-binding domain

Based upon the above results, we addressed whether homology modelling with the previously solved structure of the E2F-4/DP-2 heterodimer (Zheng *et al*., 1999) would allow the two domains to adopt a structure consistent with DNA binding. Thus, we built two models in which either domain 1 or 2 simulated the E2F partner. Based upon the conservation in the heterodimerization contacts and residues involved in DNA backbone and base contacts (Figure 5a), both approaches gave rise to modelled structures on DNA (Figure 5b). However, since domain 2 exhibits greater similarity to the E2F DNA-binding domain than domain 1 (Figure 5a), it is more likely that model 1 in which domain 2 acts as the surrogate E2F subunit in the E2F/DP heterodimer reflects the structure of the E2F-7 DNA-binding domain.

DNA binding and transcription regulating properties of E2F-7

The homology model predicted that E2F-7 possesses the capacity to bind to DNA. We tested this idea by assessing the ability of *in vitro* translated E2F-7 to bind in a sequence-specific fashion to the E2F-binding site. E2F-7 could specifically bind to the E2F recognition site (Figure 6a), and the DNA-binding complex was supershifted by the anti-HA antibody, demonstrating E2F-7 in the DNA-binding complex. E2F-1 DNA-binding activity and luciferase served as the positive and negative controls.

We explored the transcription regulating properties of exogenous E2F-7 on a panel of E2F reporter constructs, investigating both its repressing and activating properties. As a Gal4-E2F-7 hybrid protein, E2F-7 failed to exhibit significant repression activity when the response of the pSV40-Gal4 reporter was measured, in contrast to the repression of Gal4-pRb, which possessed marked repression activity (data not shown; Chan *et al*., 2001). Similarly, there was insignificant response of the reporters in the presence of E2F-7 when activation was measured (Figure 6b, and data not shown).

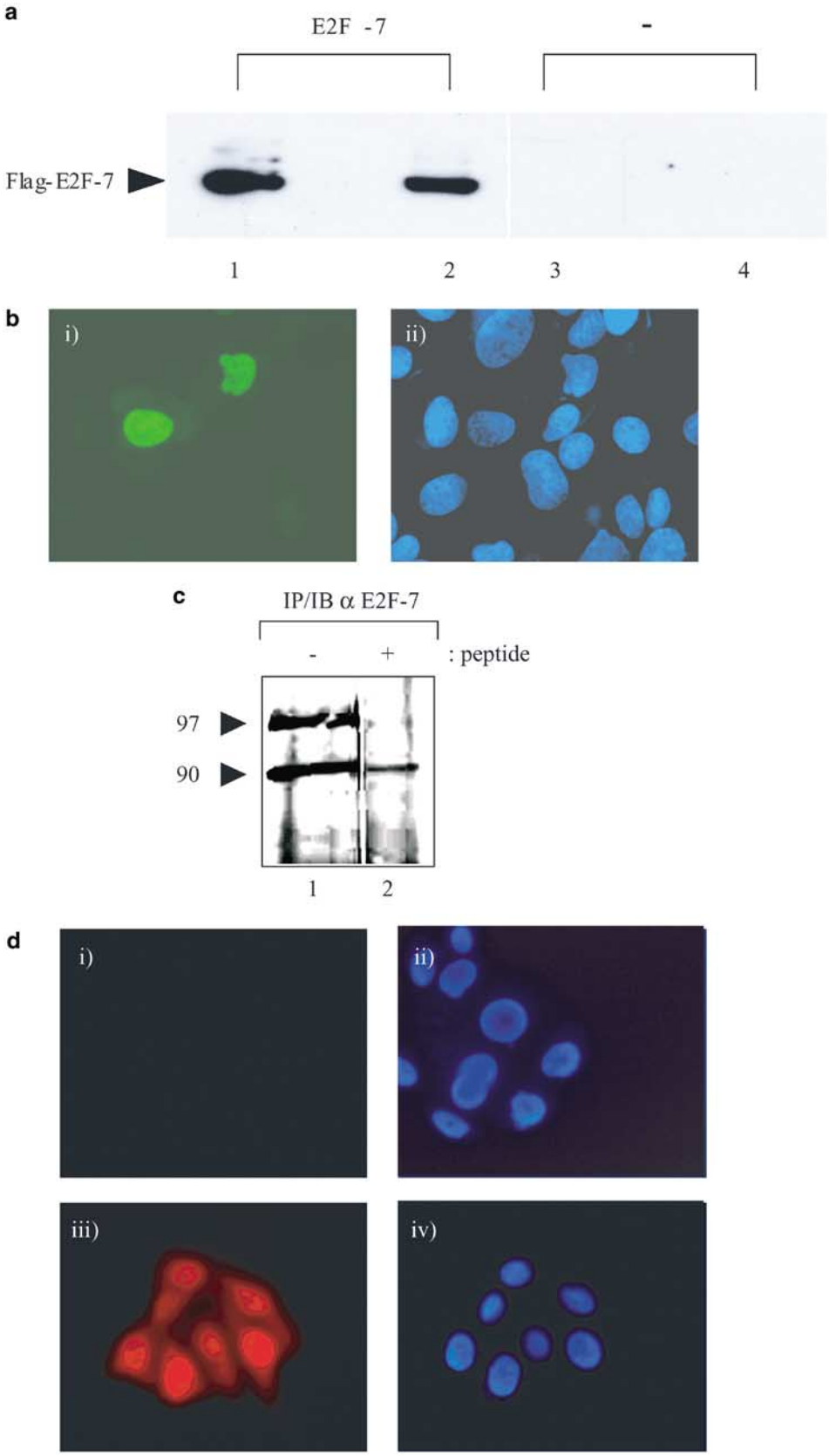
Based upon these results, together with the intrinsic DNA-binding activity of E2F-7, we reasoned that E2F-7 may modulate the activity of other E2F proteins. To explore this idea, we coexpressed E2F-7 with E2F-1, in conditions were E2F-1 could efficiently activate diverse E2F genes. On the adenovirus E2A promoter, a known target gene for E2F (Bandara *et al*., 1993), we found as expected that E2F-1 induced reporter activity (Figure 6c). Surprisingly, while E2F-7 alone failed to activate the E2A promoter, coexpression of E2F-7 with E2F-1 reduced E2A activity in a fashion that reflected the level of cotransfected E2F-7 (Figure 6c). Similar results were obtained on additional E2F-responsive promoters, such as APAF1, where E2F-7 could override activation by E2F-1 (Figure 6d). Overall, these results argue that E2F-7 can influence the activity of other E2F subunits, such as E2F-1.

To assess whether the integrity of the domains 1 and 2 was required to overcome E2F-1 activation, we used the two E2F-7 mutant derivatives that were altered in each of the conserved RRXYD motifs, called R185A and R355A, to RAXYD (Figure 7a). By gel retardation, both R185A and R355A were compromised in DNA-binding activity (Figure 6a). The two mutant derivatives were expressed after transfection into U2OS cells where, compared to wild-type E2F-7, both R185A and R355A failed to overcome activation of the E2A promoter by E2F-1 (Figure 7c), thus arguing that the integrity of both DNA-binding domains is required for E2F-7 to block activation by E2F-1. Both mutants were similarly expressed in transfected cells (Figure 7b).

Cell cycle control by E2F-7

We investigated the growth-regulating properties of E2F-7 in asynchronously growing U2OS cells, a cell-type that has been used to study the cell cycle properties of E2F subunits. As reported previously (Stevens *et al*., 2003), the introduction of E2F-1 into U2OS cells results in increased levels of apoptosis, together with a concomitant increase in G1 and reduction in G2/M cells (Figure 8a). In contrast, the introduction of E2F-7 caused U2OS cells to undergo a very significant induction of G1 cells with a reduction in G2/M phase cells; this effect was titratable as increasing the level of E2F-7 caused a proportional increase in the amount of cells in G1 (Figure 8a).

Figure 1 Sequence of E2F-7 and comparison to E2F family members. (**a**) Complete sequence of human E2F-7 (910 residues) using single letter amino-acid code. The two DNA-binding domains, domains 1 (orange; residues 160–220) and domain 2 (red; 270–375) are indicated. (**b**) Line diagram of E2F-7 in comparison to other mammalian E2F and DP family members, including *Arabidopsis* E2L. The conserved domains are indicated; NLS, nuclear export signal (NES), DNA-binding domains 1 and 2 (DB1 and DB2). (**c**) Comparison of the two DNA-binding domains in E2F-7 with other E2F family members, including *Arabidopsis* E2L1. Residues conserved across all E2F proteins indicated in blue, and residues conserved within the mammalian E2F family are indicated in red


a
E2F -7
-
Flag-E2F-7
1
2
3
4
b
i)
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c
IP/IB α E2F-7
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97
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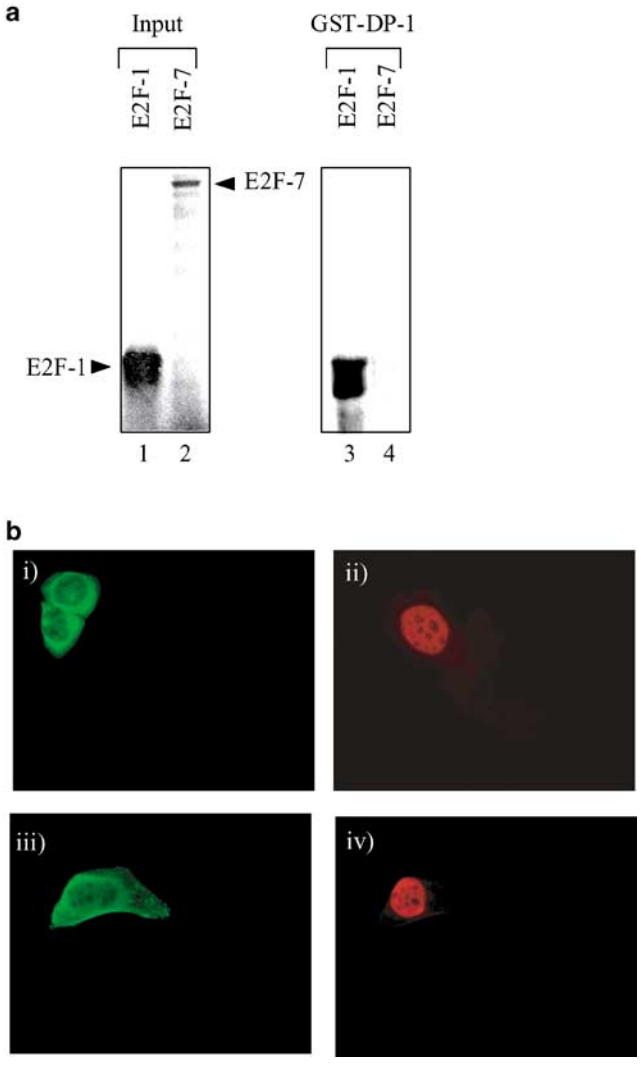

Figure 3 E2F-7 fails to interact with a DP partner. (**a**) *In vitro* binding assay between E2F-7 and DP-1: purified GST-DP-1 (1 μg) was incubated with *in vitro* translated E2F-1 or E2F-7 and the level of bound E2F-1 and E2F-7 (track 3 and 4) assessed as described. Track 1 and 2 show 10% of the input protein. (**b**) Coexpression of E2F-7 and DP-1 in U2OS cells: Expression vectors for Flag-E2F-7 (5 μg; ii and iv) or DP-1 (5 μg; i and iii) were transfected alone (i and ii) or together (iii and iv) and immunostained as described. iii) and iv) show the same cell stained for DP-1 (iii) or Flag-E2F-7 (iv)

Figure 2 Properties of E2F-7. (**a**) U2OS cells were either transfected with pFlag-E2F-7 (5 μg; tracks 1 and 2) or empty vector (5 μg; tracks 3 and 4) as described and immunoblotted with the anti-Flag M2 antibody. Ectopic E2F-7 resolves as a 95 000 molecular weight polypeptide. (**b**) Ectopic E2F-7 is a nuclear protein: U2OS cells were transfected with pFlag-E2F-7 (5 μg) and immunostained as described. (**c**) HeLa cell extracts were immunoprecipitated with the peptide affinity purified anti-E2F-7 peptide antibody and the immunoprecipitates immunoblotted with the same antibody as described in the presence of E2F-7 (10 μM; track 2) or an unrelated (10 μM; track 1) peptide. The 97 and 90 kDa E2F-7 polypeptides are indicated. (**d**) Endogenous E2F-7 resolved in U2OS cells by immunostaining with the peptide affinity purified anti-E2F-7-peptide antibody (i and iii) in the presence (i) or absence (ii) of competing peptide. DAPI staining (ii and iv) is shown for each representative field

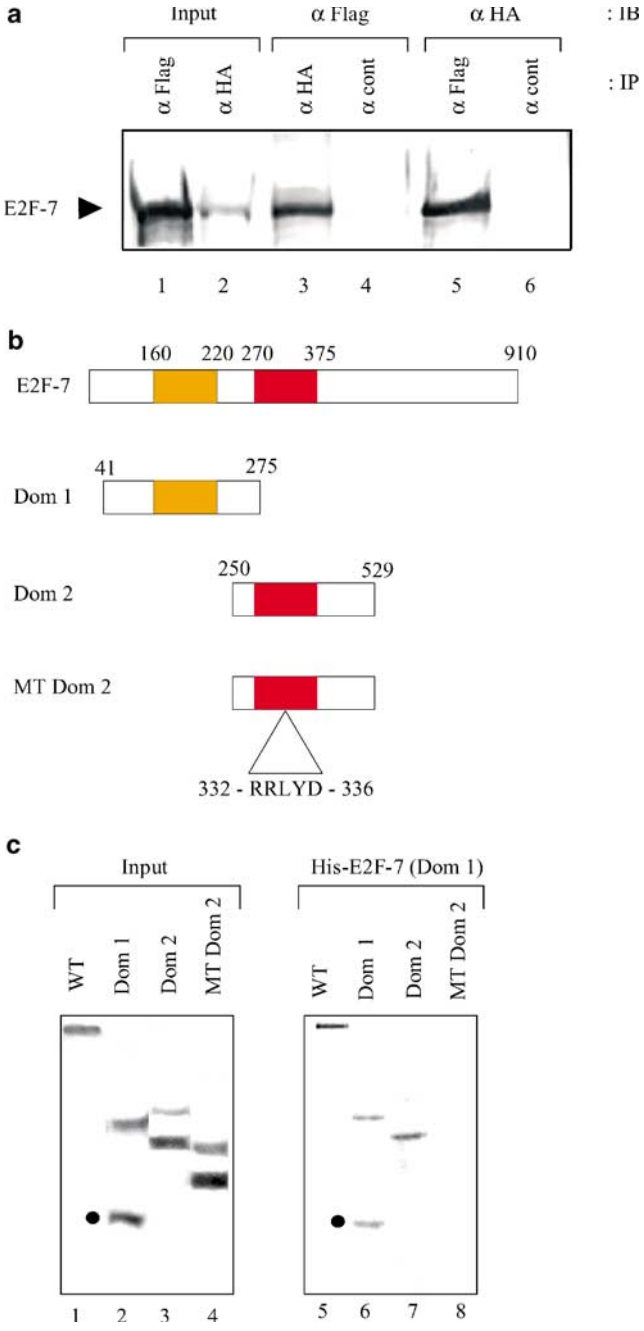

Figure 4 Protein-binding properties of E2F-7. (**a**) U2OS cells were transfected with expression vectors encoding pFlag-E2F-7 or pHA-E2F-7 (3 μg of each) as described and immunoprecipitated with either anti-Flag (track 3), anti-HA (track 5) or a control unrelated antibody (tracks 4 and 6) and immunoblotted with the opposite antibody as indicated. Tracks 1 and 2 indicate the input levels of Flag-E2F-7 and HA-E2F-7 in the crude extracts. (**b**) E2F-7 derivatives used in protein-binding assays. In MT Dom2 the conserved RRLYD motif is deleted. (**c**) His-E2F-7/domain 1 (residues 41–275) was incubated with *in vitro* translated wild-type E2F-7 (track 5), domain 1 (track 6), domain 2 (track 7) or MT domain 2 (track 8) and treated as described. Tracks 1–4 show 10% of each of the input proteins, and • indicates the domain 1 polypeptide

To determine whether cell cycle arrest by E2F-7 required the integrity of domains 1 and 2, we investigated the cell cycle-regulating properties of

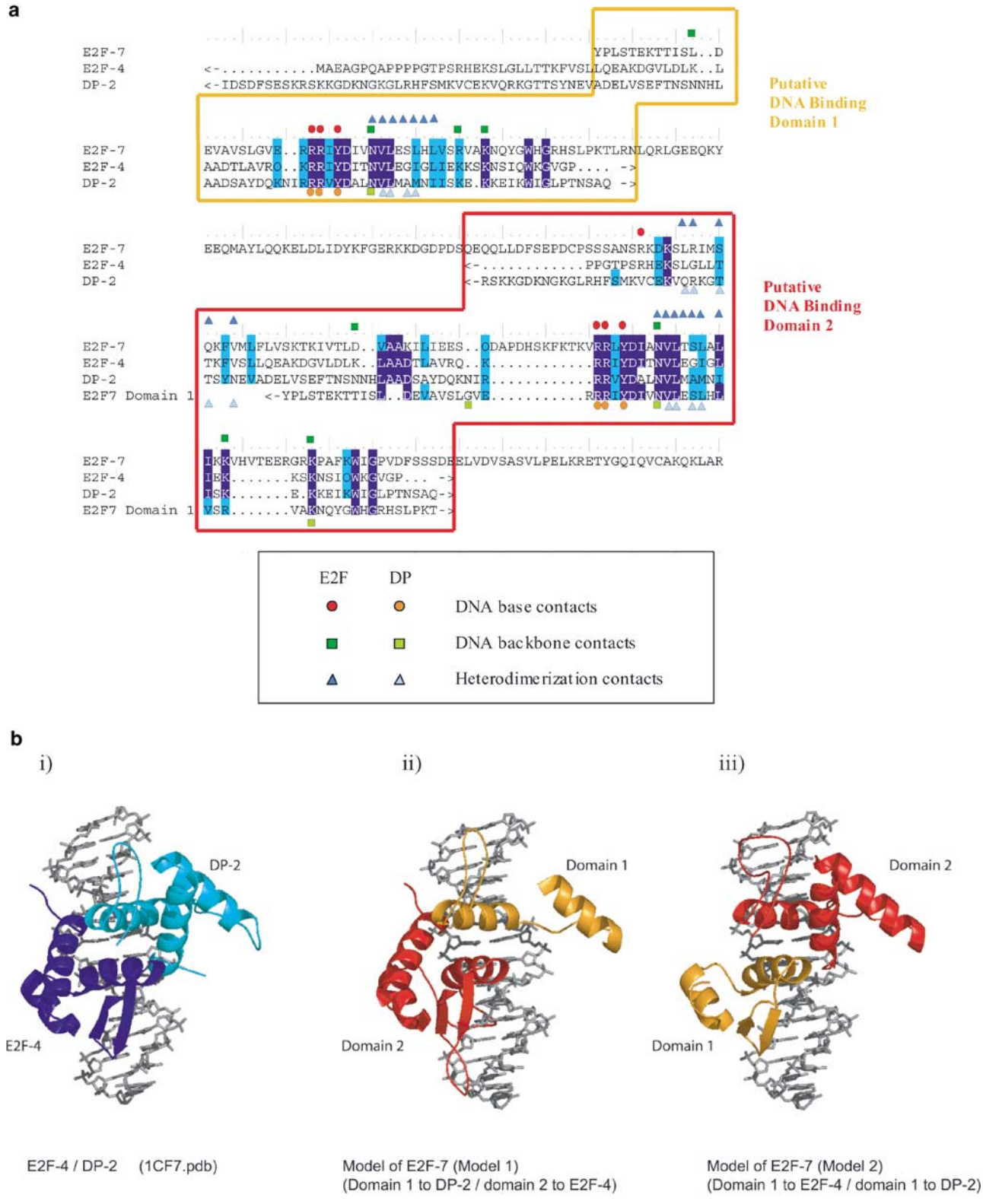

Figure 5 Structural model of domains 1 and 2 in E2F-7 DNA binding. (**a**) Sequence of E2F-7 domains 1 (orange) and 2 (red) compared to E2F-4 and DP-2, detailing conserved residues in E2F-7 involved in DNA base, DNA backbone and heterodimerization contacts with E2F-4 and DP-2 (key shown at bottom of sequences). Contact residues in E2F-4 and DP-2 taken from Zheng *et al.* (1999). (**b**) (i) The structure of the E2F-4/DP-2 heterodimer (taken from Zheng *et al.*, 1999), indicating E2F-4 (blue) and DP-2 (turquoise). (ii) Model depicting the interaction between domains 1 (orange) and 2 (red) in E2F-7 DNA binding based upon homology modelling with the solved structure of the E2F-4/DP-2 heterodimer shown in (**b** (i)). In (**b** (ii)), domain 2 (red) is modelled as the E2F partner, whereas in (iii) domain 1 (orange) is modelled as the E2F partner

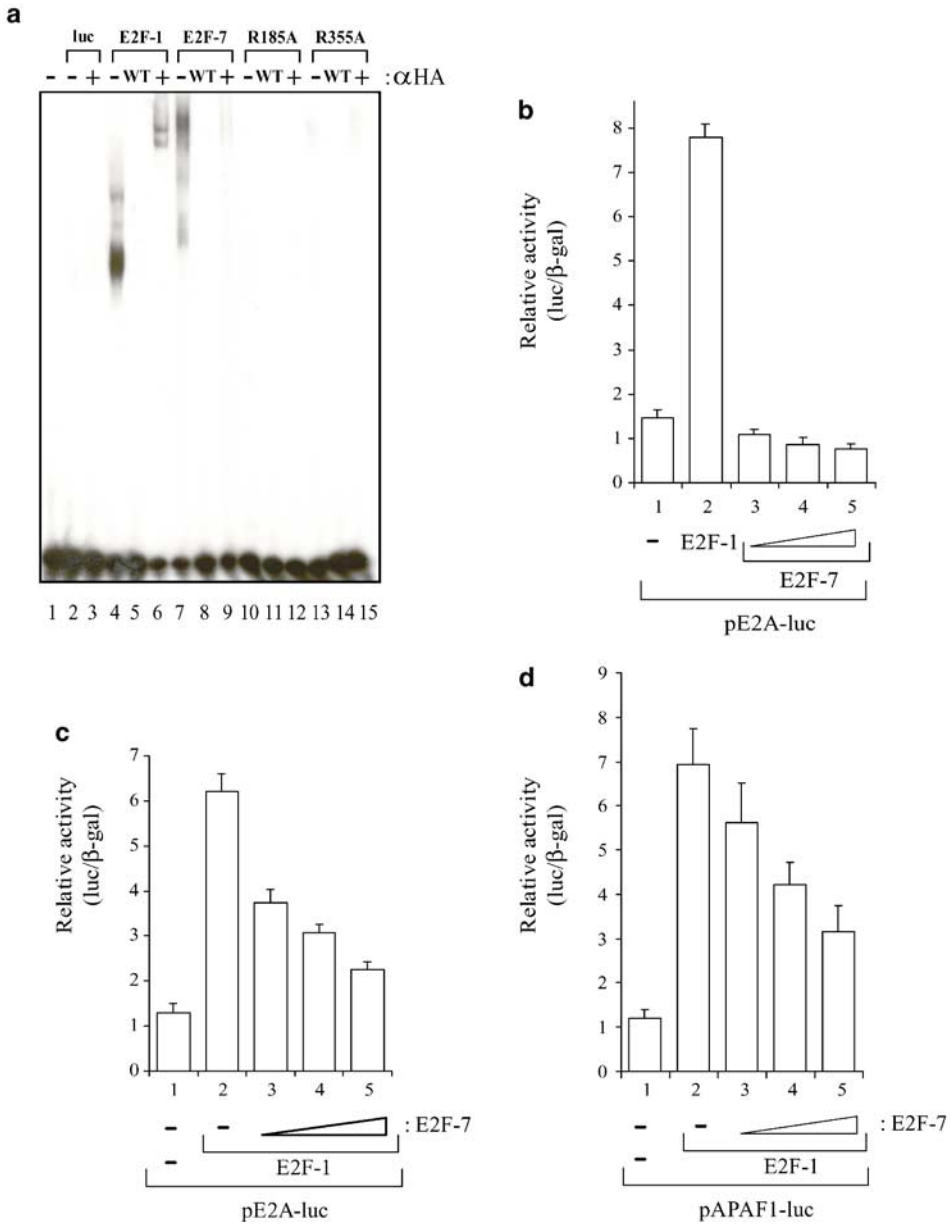

Figure 6 DNA binding and transcription regulating properties of E2F-7 (**a**) *In vitro* translated luciferase (tracks 2 and 3), HA-E2F-1 (tracks 4–6), HA-E2F-7 (tracks 7–9), HA-R185A (tracks 10–12) or HA-R355A (tracks 13–15) were assessed for DNA binding on the E2F site taken from the E2A promoter as described. Track 1 shows the probe alone; – and wt indicates the presence of competing (nonradiolabelled) mutant or wild-type E2F site (1000-fold excess) and + the presence of anti-HA antibody. (**b**) U2OS cells were transfected with expression vectors encoding E2F-1 (100 ng) or E2F-7 (1, 2 or 3 μg) as indicated together with the pE2A-luc reporter construct (1 μg) and pCMV-β-gal (1.0 μg). The results represent the ratio of luciferase to β-gal activity. (**c**, **d**) U2OS cells were transfected with expression vectors encoding E2F-1 (1 μg) together with increasing amounts of E2F-7 (1, 2 or 3 μg, respectively), together with the pE2A-luc (**c**) or pApaf1-luc (**d**) reporter construct (1 μg) and pCMV-β-gal (1.0 μg). The results represent the ratio of luciferase to β-gal activity

R185A and R355A. In contrast to wild-type E2F-7, neither R185A nor R355A had a significant effect on cell cycle progression in U2OS cells (Figure 8b), suggesting that cell cycle arrest by E2F-7 requires the integrity of domains 1 and 2. In this respect, there is a clear correlation between E2F-7 DNA-binding activity, regulation of transcription and cell cycle arrest.

Discussion

E2F-7 contains two DNA-binding domains

The properties of E2F-7 indicate that it is a unique member of the E2F family. Specifically, two stretches of protein sequence, referred to as domains 1 and 2,

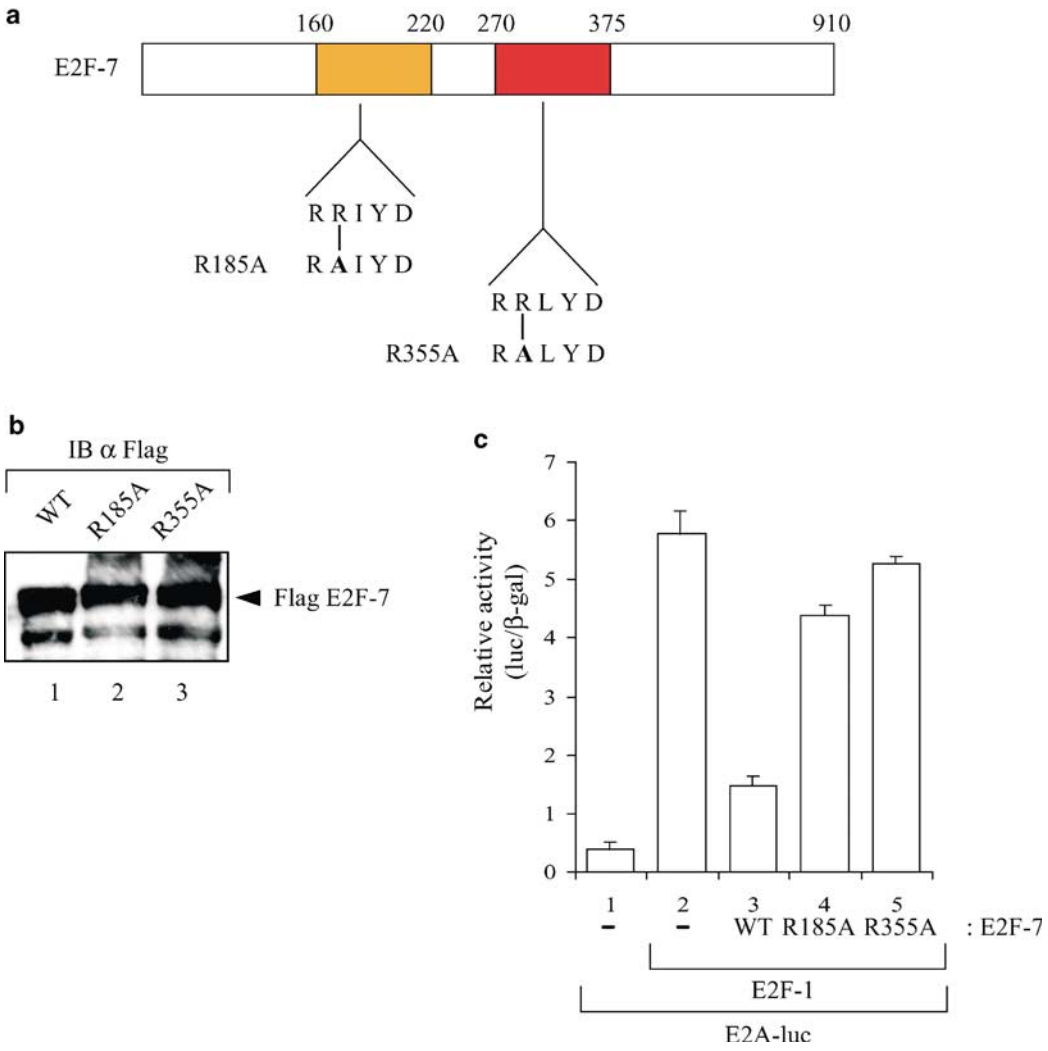

Figure 7 (**a**) Diagrammatic presentation of the two E2F-7 mutant derivatives, R185A and R355A. (**b**) Expression level of E2F-7, R185A and R355A after transfection (10 μg) into U2OS cells. (**c**) U2OS cells were transfected with expression vectors encoding E2F-1 (100 ng) together with E2F-7, R185A or R355A (3 μg) as indicated together with the pE2A-luc reporter construct (1.0 μg) and pCMV-β-gal (1.0 μg). The results represent the ratio of luciferase to β-gal activity

contain similarity to the E2F DNA-binding domain. In this respect E2F-7 differs from all other members of the mammalian E2F family characterized to date that contain a single DNA-binding domain (Stevens and La Thangue, 2003). Other notable features, such as a C-terminal activation domain and pocket protein-binding motif, are also absent in E2F-7.

Sequence comparison with members of the E2F family, including the DP heterodimeric partner, indicated that domain 2 contains more extensive similarity to the E2F/DP core DNA-binding domain compared to domain 1, although domain 1 is nevertheless well conserved (Figure 5). Using these domains, we homology modelled E2F-7 on the solved structure of the E2F-4/DP-2 DNA-binding heterodimer (Zheng *et al*., 1999). The modelled E2F-7 structure closely resembled the solved E2F-4/DP-2 structure, implying that domains 1 and 2 in E2F-7 may undergo interactions in a fashion that mimics the DNA-binding structure of the E2F/DP heterodimer. It is consistent with this idea that *in vitro* E2F-7 exhibits DNA-binding activity and fails to associate with a DP partner. Moreover, our evidence for the association between distinct E2F-7 molecules in cells implies that the DNA-binding form is mediated through the intermolecular interactions between E2F-7 molecules, results that are generally consistent with other recent studies on E2F-7 (De Bruin *et al*., 2003; Di Stefano *et al*., 2003).

In this respect, the properties of E2F-7 resemble those of several plant E2F-like proteins recently identified in *Arabidopsis* (Kosugi and Ohashi, 2002). The plant E2Fs, referred to as E2L1, 2 and 3, contain two separate DNA-binding domains that bind to DNA in the absence of a DP partner (Kosugi and Ohashi, 2002). While a role for the plant E2Fs has yet to be defined, it has been suggested that they function in transcriptional repression. Our results indicate that although E2F-7 lacks the

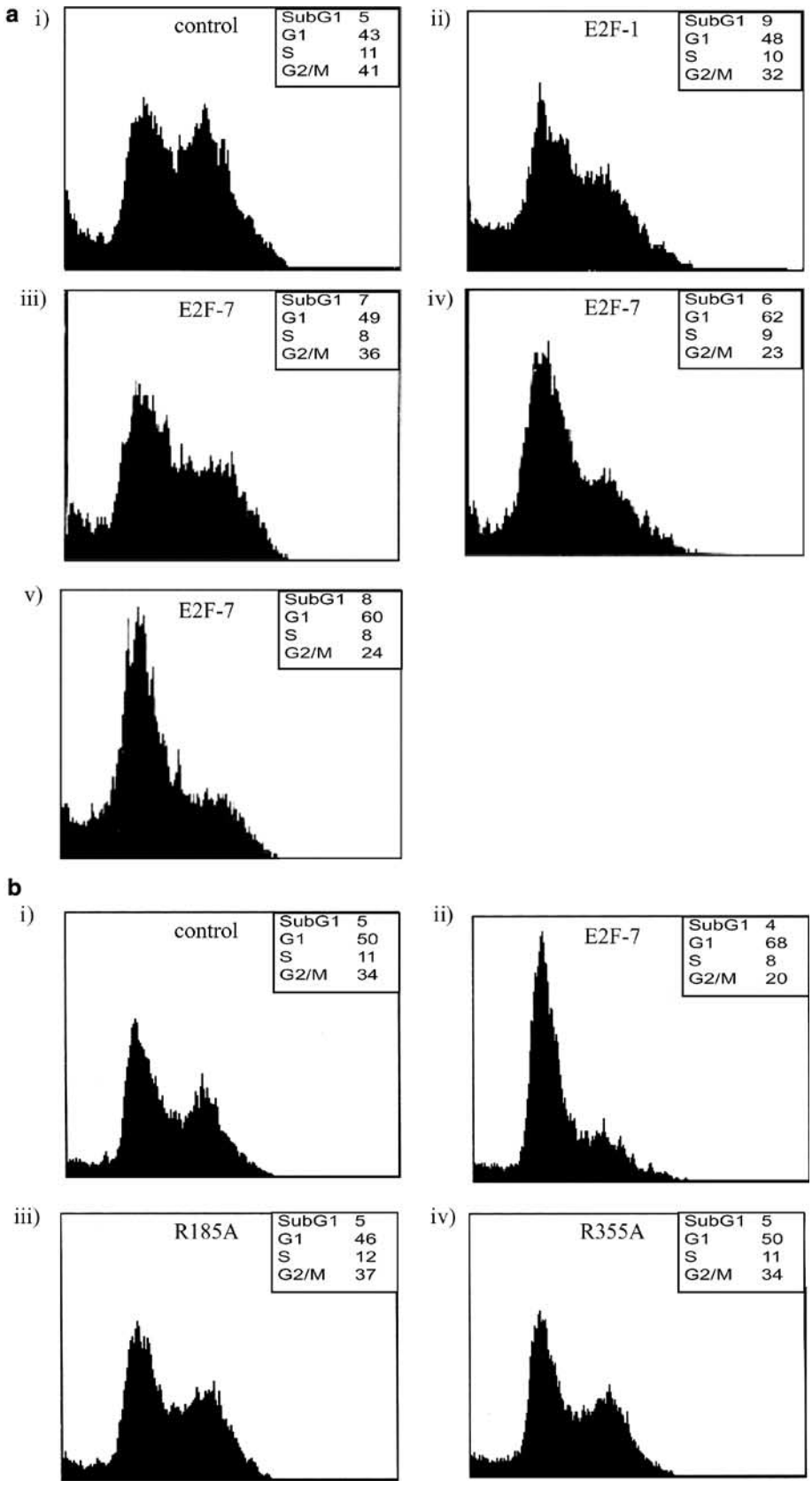

Figure 8 Growth-regulating properties of E2F-7. (**a**) Flow cytometry on U2OS cells that had been transfected with expression vectors encoding E2F-1 (ii; 5 μg), E2F-7 (iii–v; 1, 5 or 10 μg) or pCDNA (ii; 10 μg) as indicated. Flow cytometry was performed as described, and the level of sub-G1, G1, S and G2/M phase cells are indicated in parentheses. (**b**) Flow cytometry on U2OS cells was performed as described in (**a**) with expression vectors (10 μg) encoding E2F-7 (ii), R185A (iii), R355A (iv) or pCDNA (i)

intrinsic ability to repress transcription, it is nevertheless capable of modulating transcriptional activation driven by other E2F subunits. For example, coexpressing E2F-7 with E2F-1 lowered the level of activation by E2F-1. Although the mechanism involved in mediating this effect requires further investigation, the fact that this inhibitory activity requires the integrity of domains 1 and 2 implies that the inhibition requires E2F-7 DNA-binding activity.

Another feature that differentiates E2F-7 from other E2F subunits is cell cycle arrest through the induction of G1 cells and a reduction of G2/M phase cells. Other members of the family can stimulate cell cycle progression or apoptosis. For example E2F-1 can induce apoptosis (Qin *et al*., 1994; Shan and Lee, 1994) whereas the combined ablation of E2F-1, E2F-2 and E2F-3 results in cell cycle arrest (Wu *et al*., 2001). Similarly, E2F-4 and E2F-5 exhibit growth promoting (Morris *et al*., 2000), or inhibitory properties when bound to pocket proteins (Gaubatz *et al*., 2000). In contrast, E2F-6 assembles with a complex of proteins involved in transcriptional repression, thereby preventing the activation of E2F-responsive genes (Ogawa *et al*., 2002). In a similar fashion to the transcription regulating properties, E2F-7 requires domains 1 and 2 to cause the induction of G1 cells, again arguing that DNA binding is required for cell cycle arrest.

The molecular rationale for the presence of two DNA-binding domains in E2F-7 requires further investigation. However, there are examples of other transcription factors, such as Oct1, with separable domains that contribute to DNA-binding activity. In Oct1, the Pou-specific and Pou-homeo domains act together in selecting DNA recognition sites (Klemm and Pabo, 1996). In the centromere-binding protein B a tandem repeat of two DNA-binding domains is required for binding activity (Lee *et al*., 1997) and in the *Escherichia coli* RepE54 protein a tandem repeat of two winged helix domains arranged with pseudo twofold symmetry contributes to DNA binding (Komori *et al*., 1999).

Our modelling predictions argue that the interactions between the two domains allows E2F-7 DNA-binding activity, and therefore overcomes the requirement for a DP partner. Perhaps a key role for E2F-7 will be found to be in situations where the level of DP-1 is limiting. Towards the completion of S phase, DP-1 becomes phosphorylated by cyclinA/cdk2, lowering the DNA-binding activity of the E2F/DP heterodimer which, ultimately, may contribute to reducing the activity of E2F-responsive genes (Krek *et al*., 1994). It is in conditions such as these that we may expect the activity of E2F-7 to come into play. Future experiments will clarify this possibility.

Materials and methods

Isolation of the human E2F-7 cDNA

The Genbank database was searched with the DNA-binding domain of E2F-1 using the BLAST programme A 2.0 kb clone showing homology to the E2F DNA-binding domain consensus sequence was identified and thereafter obtained from the IMAGE consortium (Genbank accession no. BC0174810). Further database searches revealed an overlapping cDNA clone (Genbank accession no. AK097677) that was used to design primers and amplified using cDNA from cultured HeLa cells as a template. cDNA was prepared from mRNA isolated from HeLa cells using the Marathon cDNA isolation kit (Clontech). The human 3′ ORF of E2F-7 was amplified with the primers 7F: CCGTTGCTCCTTTCCCTGTCCTCTCTG and 7R: GGAAATCAGATGATTGATGGTGGTGGG using Advantage HF2 polymerase (BD Biosciences). The PCR product was subcloned into PCR2.1 (Invitrogen). The resulting 1.5 kb clone was ligated to the IMAGE clone using a unique *Ssp*I site in the overlapping region. The full-length 3.0 kb E2F-7 coding sequence was subsequently cloned in frame into p3xCMV FLAG-7.1 (Sigma).

Site-directed mutagenesis

The following primers were used: R155A: 5′ AGT CTT GGT GTG GAA AGG AGA GCC ATC TAT GAC ATT GTA AAT CTG and R355A: 5′ AAA ACA AAG GTA CGA GCC CTC TAT GAC ATA GCC AAT GTT CTG. pFlag-E2F-7 together with one of the above primers was amplified by PCR using reagents and protocol supplied with Stratagene's quick change multisite-directed mutagenesis kit. The amplified products were then digested with Dpn1, and transformed into XL10-GOLD ultracompetant cells, sequenced by Lark Technologies and maxiprepped with Qiagen plasmid maxiprep kit.

Cell culture and transfection

U2OS cells were grown under standard conditions in DMEM supplemented with 10% foetal calf serum (FCS). For immunoblotting 10 cm plates were transfected with 5 μg of pFlag-E2F-7 using the standard calcium phosphate precipitation method. Protein extracts were prepared 48 h after transfection by scraping the cells and re-suspending the cell pellet in 80 μl of TNN buffer (50 mM Tris, pH 7.4, 0.5% NP40, 250 mM NaCl, 5 mM EDTA, 10% glycerol) at 4°C for 30 min and then centrifuged at 14 000 r.p.m. for 20 min at 4°C. Supernatants were assayed for total protein using the Bradford method (Biorad) according to the instructions provided. In all, 70 μg of total protein was analysed by SDS–PAGE and Western blotting.

Immunoblotting and immunoprecipitation

Immunoblotting analysis was performed according to standard procedures using M2 monoclonal anti-Flag (Sigma) or anti-E2F-7 peptide antibody raised against a peptide representing residues 751–765. The antipeptide antibody was affinity purified as previously described (Stevens *et al*., 2003). For immunoprecipitation, cells were lysed in TNN buffer and incubated for 24 h at 4°C with the appropriate antibody bound to protein A–sepharose beads. Proteins were released and resolved by SDS–PAGE, followed by immunoblotting. For peptide competition, E2F-7 peptide was added ar 10 μM.

Luciferase assays

The pE2A-luc construct was generated by subcloning the 346 bp *Bgl*II/*Xho*I Ad5 DNA fragment containing 62 bp coding for the first leader segment and 284 bp upstream of the cap site of the E2A promoter (La Thangue and Rigby, 1987) into pGL3-Basic (Promega). pApaf1-luc has been previously described (Moroni *et al*., 2001).

For reporter assays U2OS cells were grown in 60 mm dishes and transfected with 1 μg of luciferase reporter plasmid, 1 μg of pCMVβGal plasmid as an internal control and the indicated amounts of the expression plasmids pFlag-E2F-7, pCMV-HA-E2F-1, pCMV-HA-DP1. DNA levels were brought up to a total of 10 μg by the addition of pcDNA3. Transfected cells were harvested by gentle scraping into 300 μl of luciferase assay lysis buffer (Promega). Lysates were transferred and centrifuged at 14 000 r.p.m. for 10 min at 4°C. The supernatants were used for luciferase and β-galactosidase activity assays. All were performed in duplicate and were normalized to β-galactosidase expression. The data presented represent a typical example taken from three independent experiments.

Immunostaining

U20S cells were seeded onto 13 mm borosilicate glass coverslips and transfected with 1 μg of pFlag-E2F-7. At 48 h post-transfection the cells were washed twice with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.1%Triton X-100 and blocked with PBS containing 10% foetal calf serum (FCS). The Flag M2 antibody was incubated for 30 min at a dilution of 1 : 1000 in 1% FCS/PBS. Secondary anti-mouse Alexa 594 antibody was from Molecular Probes (Eugene, OR, USA). Immunostaining with the peptide affinity purified anti-E2F-7 peptide antibody was performed as described (Stevens and La Thangue, 2003). Cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Images were captured using an Olympus BX60 system microscope with Hamamatsu C4742-95 digital camera. Images were analysed using Improvision Openlab digital image software.

In vitro *protein binding*

Either the GST or His-tagged protein (1 μg) bound to beads was incubated in 50 μl 20 mM Tris, pH 8.0, 100 mM NaCl, 1% NP40 containing protease inhibitors. E2F-1, E2F-7, Dom1, Dom2 or MT Dom2 in pCDNA3 were *in vitro* translated and, after incubation with the *in vitro* translate the beads were washed five times in the same buffer and analysed by SDS–PAGE.

Gel retardation

Gel retardation was carried out in 20 μl reactions containing 2 μg salmon sperm DNA, reaction buffer (10 mM Hepes, pH 7.9, 100 KCl, 4 mM EDTA, 4% Ficoll, 1 mM DTT) and *in vitro* translated proteins. Where complexes were identified by antibody shifts 1 μl of HA11 antibody (Babco) was added to the reaction. Reactions were incubated for 15 min, then radiolabelled probe was added and incubated for a further 15 min followed by electrophoresis on a 4% polyacrylamide gel run at 4°C. The E2F-binding site probe contained the distal E2F site from the E2A promoter (La Thangue and Rigby, 1987). Proteins were *in vitro* translated using the TNT coupled reticulocyte lysate kit (Promega).

Flow cytometry

U2OS cells seeded in 10 cm plates were transfected with 2, 5 and 10 μg of pFlag-E2F-7, 10 μg of pCMV-R185A or pCMV-R355A, 5 and 10 μg of pCMV HA-E2F-1 and pCMV HA-DP1 together with 8 μg of pCMV-CD20. At 48 h after transfection cells were harvested in cell dissociation buffer (Sigma) and incubated with FITC-conjugated CD20 antibody (Becton-Dickinson) to identify the transfected cell population. The cells were then washed in PBS and fixed overnight at 4°C. Cells were sorted on a FACScan cell sorter (Becton-Dickinson) and analysed using the Cell Quest Software package.

Protein modelling

The putative DNA-binding domains from the E2F-7 sequence were aligned with the E2F-4 and DP-2 DNA-binding domains using the ClustalW program. This alignment was used as the input for the SWISS-MODEL protein modelling server (Schwede *et al*., 2003) using the PDB structure file (1CF7) as the template.

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