Id helix–loop–helix proteins inhibit nucleoprotein complex formation by the TCF ETS-domain transcription factors

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The Id subfamily of helix–loop–helix (HLH) proteins plays a fundamental role in the regulation of cellular proliferation and differentiation. Id proteins are thought to inhibit differentiation mainly through interaction with other HLH proteins and by blocking their DNA-binding activity. Members of the ternary complex factor (TCF) subfamily of ETS-domain proteins have key functions in regulating immediate-early gene expression in response to mitogenic stimulation. TCFs form DNA-bound complexes with the serum response factor (SRF) and are direct targets of MAP kinase (MAPK) signal transduction cascades. In this study we demonstrate functional interactions between Id proteins and TCFs. Ids bind to the ETS DNA-binding domain and disrupt the formation of DNA-bound complexes between TCFs and SRF on the *c-fos* serum response element (SRE). Inhibition occurs by disrupting protein–DNA interactions with the TCF component of this complex. *In vivo*, the Id proteins cause down-regulation of the transcriptional activity mediated by the TCFs and thereby block MAPK signalling to SREs. Therefore, our results demonstrate a novel facet of Id function in the coordination of mitogenic signalling and cell cycle entry.

Keywords: ETS-domain proteins/helix–loop–helix proteins/Ids/TCFs/transcription factor

Introduction

The ternary complex factors (TCFs) comprise a subfamily of ETS-domain transcription factors (reviewed in Treisman, 1994). These proteins form ternary complexes with the serum response factor (SRF) and serum response elements (SREs) found in immediate-early genes such as *c-fos* (reviewed in Treisman, 1994; Cahill *et al.*, 1996). Three mammalian TCFs (Elk-1, SAP-1 and SAP-2/ERP/Net) have been identified (Rao *et al.*, 1989; Dalton and Treisman, 1992; Giovane *et al.*, 1994; Lopez *et al.*, 1994; Price *et al.*, 1995). Each protein shares four major regions of primary amino acid sequence similarity. The ETS DNA-binding domain is located at the N-terminus of the protein and together with a second conserved region, the B-box,

is sufficient for ternary complex formation with SRF and the *c-fos* SRE (Dalton and Treisman, 1992; Janknecht and Nordheim, 1992; Marais *et al.*, 1993; Giovane *et al.*, 1994; Janknecht *et al.*, 1994; Price *et al.*, 1995). The C-terminal domain (C-box) is a target for phosphorylation by MAP kinases (MAPKs) and acts as an autonomous transcriptional activation domain in a heterologous context (reviewed in Treisman, 1994). The fourth conserved region (D-domain) is located immediately N-terminal from the C-box and acts as a MAPK-targeting domain (Yang *et al.*, 1998a,b). Ternary complex formation by TCFs requires both protein–DNA interactions mediated by the ETS-domain (Dalton and Treisman, 1992; Janknecht and Nordheim, 1992) and protein–protein interactions with SRF mediated by the B-box (Shore and Sharrocks, 1994). In the case of Elk-1 and SAP-1, ternary complex formation is stimulated by MAPK phosphorylation of the TCFs (Gille *et al.*, 1992, 1995a,b; Shore *et al.*, 1996).

The ternary TCF–SRF–SRE complex plays a pivotal role in the activation of immediate-early genes such as *c-fos* in response to a series of mitogenic and stress stimuli (reviewed in Cahill *et al.*, 1996). The rapid induction of *c-fos* and other immediate-early genes is followed by a rapid shut-off back to basal levels (reviewed in Herschman, 1991). Genes encoding members of the Id family of helix–loop–helix (HLH) proteins also represent immediate-early genes which are markedly and rapidly induced by serum and other mitogenic stimuli (Christy *et al.*, 1991; Deed *et al.*, 1993; Barone *et al.*, 1994; Hara *et al.*, 1994; reviewed in Norton *et al.*, 1998). The prototype, Id1, was originally identified as an inhibitor of DNA binding by basic helix–loop–helix (bHLH) proteins (Benezra *et al.*, 1990). Subsequently, three further genes were identified that encode the related proteins Id2, (Sun *et al.*, 1991; Biggs *et al.*, 1992), Id3 (Christy *et al.*, 1991; Deed *et al.*, 1993) and Id4 (Riechman *et al.*, 1994). The Id proteins contain functional HLH dimerization motifs but lack the DNA-binding basic region found in the bHLH proteins. Id-mediated inhibition of DNA binding by bHLH proteins is thought to occur by sequestration of these proteins into inactive dimers which cannot bind DNA due to the absence of a basic region in the Id proteins (Benezra *et al.*, 1990; Sun *et al.*, 1991; reviewed in Norton *et al.*, 1998).

In addition to their association with bHLH transcription factors, Id proteins have been shown to interact with the non-HLH proteins, retinoblastoma protein (pRB) (Iavarone *et al.*, 1994) and MIDA1 (Shoji *et al.*, 1995). In the case of pRB, binding of Id2 to the pocket domain results in the inhibition of RB function in inducing cell cycle arrest. Several lines of evidence therefore show that Id proteins function to promote cell cycle entry, to stimulate proliferation and to block cellular differentiation (reviewed in Norton *et al.*, 1998).

Interactions between several ETS-domain and bHLH

Fig. 1. The TCFs interact with Id2 through the ETS-domain. **(A)** Schematic illustration of the domain structure of the proteins used. Numbers indicate the positions of the N- and C-terminal residues with respect to the full-length proteins. **(B)** GST pull-down analysis of full-length TCFs with GST (lanes 4–6) and a GST-Id2 fusion protein (lanes 7–9). Twenty per cent of the input proteins are shown in lanes 1–3. **(C)** Mapping the Id2 interaction domain on Elk-1. GST pull-down analysis of full-length Elk-1 with GST (lane 1) and full-length or truncated Elk-1 derivatives with GST-Id2 (lanes 2–5). **(D)** Elk-1 and Id2 interact *in vivo*. **(i)** Schematic illustrating the domain structure of full-length Id2 (amino acids 1–134) containing the conserved HLH domain (represented as black boxes) and Flag-epitope-tagged full-length Elk-1 (amino acids 1–428). **(ii)** Co-immunoprecipitation of Id2 and Elk-1 from Cos-7 cells. Cells were either mock transfected with 1 µg pCMV5 and 1 µg pcDNA3 (lane 1), 1 µg of pcDNAId2 and 1 µg pCMV5 (lane 2), or 1 µg of pcDNAId2 and 1 µg of pCMVElk-1 (lane 3). A marker of bacterially purified Elk-1 protein (20 ng) is shown in lane M.

transcription factors have recently been demonstrated or inferred. Ets-1 and USF-1 interact via the ETS-domain of Ets-1 to cause cooperative activation of the HIV-1 enhancer (Sieweke *et al.*, 1998). Similarly, cooperative interactions between Ets-1 and the bHLH proteins E47 and TFE3 lead to enhanced transcription from the immunoglobulin μ heavy-chain gene enhancer (Dang *et al.*, 1998). The TCF SAP-2/Net/ERP has also been shown to interact with bHLH proteins through a HLH-like domain located immediately C-terminal to the B-box (Maira *et al.*, 1996). This domain also functions intramolecularly to inhibit DNA binding by SAP-2.

In this study, we investigated interactions between the Id HLH proteins and the TCF subfamily of ETS-domain transcription factors. We demonstrate direct interactions between Ids and the ETS DNA-binding domain of the TCFs. Binding of Ids disrupts ternary complex formation with SRF at the *c-fos* SRE *in vitro*. Furthermore, over-expression of Ids attenuates MEK-induced SRE-activation *in vivo*. Together, our results implicate Ids as novel negative regulators of TCF function and provide further insights into the regulation of proliferation and differentiation by these two classes of transcription factors.

Results

The TCF ETS-domain transcription factors interact with the HLH protein Id2

Physical interactions between several ETS-domain and bHLH transcription factors were demonstrated recently

(Maira *et al.*, 1996; Dang *et al.*, 1998; Sieweke *et al.*, 1998). As negative transcriptional regulators and, due to their up-regulation following serum stimulation (reviewed in Norton *et al.*, 1998), the Id subfamily of HLH proteins represents candidate interacting proteins that might down-regulate TCF activity. Initially, therefore, we tested whether the TCFs can interact with Id family members.

In vitro glutathione S-transferase (GST) pull-down experiments were carried out using full-length TCFs and a GST-Id2 fusion protein (Figure 1B). All three TCFs interact with Id2, although the efficiency of interaction with Elk-1 is lower than with SAP-1 and SAP-2 (Figure 1B, lanes 7–9). This suggests that the Ids interact with a conserved region of the TCFs. In order to map the Id-interaction domain on Elk-1, a series of C-terminally truncated proteins was tested for their ability to interact with GST-Id2 (Figure 1C). All of the Elk-1-truncated proteins bound to GST-Id2, indicating that the N-terminal ETS DNA-binding domain contained in Elk-1-93 was sufficient to mediate this interaction. This domain is highly conserved among the TCFs, exhibiting 72% identity among all three proteins. Further C-terminal truncations within the ETS-domain resulted in proteins that were unable to bind to Id2 (data not shown), indicating that the integrity of the ETS-domain is essential for the interaction of Elk-1 with Id2.

In order to examine whether the Id proteins interact with the TCFs *in vivo*, Cos-7 cells were transfected with expression vectors encoding Id2 and Flag-epitope-tagged Elk-1. Complexes were immunoprecipitated using anti-

Fig. 2. Interaction of TCFs with Ids *in vitro*. (A) Diagrammatic illustration of Elk-1-205, Sap-1-298 and MyoD fused to GST. The amino acids of the proteins included in the constructs are shown. GST-Elk-1-205 and GST-SAP-1-298 lack the D-domain and C-terminal transcriptional activation domain. The location of the ETS-domain and B-box of the TCFs is shown and the helices within the HLH motif in MyoD are indicated by black boxes. Bacterially expressed GST-fusion proteins were purified and equal molar concentrations used in all assays. (B) Pull-down assays of ^{35}S -labelled Id1, Id2 and Id3 by immobilized GST, GST-MyoD or GST-Elk-1-205. (C) Pull-down assay of ^{35}S -labelled Id1, Id2 and Id3 by immobilized GST, GST-MyoD or GST-SAP-1-298. Equal molar amounts of ^{35}S -labelled Id protein were used in each reaction and 20% of the input protein is shown.

Id2 antibodies followed by detection of co-precipitating Elk-1 by Western blotting using an anti-Flag antibody (Figure 1D). When both Id2 and Elk-1 were co-expressed, Elk-1 was co-precipitated with Id2 (Figure 1D, lane 3), indicating that the two proteins interact *in vivo*.

Id1, Id2 and Id3 interact with the TCFs

The ability of different Id proteins to interact with the TCFs was subsequently tested. GST-fusion proteins containing the N-terminal ends of Elk-1 and SAP-1 (including the ETS-domain; Figure 2A) were tested for their ability to bind to *in vitro* translated Id1, Id2 and Id3 (Figure 2B and C). GST-MyoD was used as a positive control as MyoD has been shown previously to bind to the Id proteins (Benezra *et al.*, 1990; Sun *et al.*, 1991). All three Id proteins bind to GST-Elk-1-205, although their relative binding efficiency varies in the order Id2>Id3>Id1 (Figure 2B). Similarly, all three Id proteins bind to GST-SAP-1-298 with a similar relative binding efficiency profile (compare Figure 2C with 2B). All three Id proteins are therefore able to bind to Elk-1 and SAP-1 *in vitro*, but of these, Id2 binds with the highest efficiency.

Mapping the TCF interaction determinants on the Id proteins

Id1, Id2 and Id3 all interact with the TCFs, albeit at a lower level with Id1. The Id proteins exhibit sequence similarity not only in their HLH domains but also in several other motifs located outside this region (reviewed in Norton *et al.*, 1998). The N-terminal region has been shown recently to play a role in the promotion of apoptosis

Fig. 3. Interaction with the TCFs requires the Id HLH domain. (A) Schematic illustration of the domain structure of the GST-Id2 proteins used in this study. The black boxes represent the two helices of the HLH domain. Numbers indicate the position of the N- and C-terminal residues with respect to the full-length Id2 protein. (B and C) Mapping the SAP-1- and E47-interaction domains on Id2. GST pull-down analysis of ^{35}S -labelled SAP-1 (1–156) (B) and ^{35}S -labelled E47 (C) with either full-length GST-Id2 (lane 2) or truncated GST-Id2 derivatives (lanes 3–8). Equal molar amounts of each GST-fusion protein were used in each reaction and 20% of each input protein is shown (lane 1).

induced by Id2 (Florio *et al.*, 1998). In order to determine which of these domains are required for interaction with the TCFs, we used a GST pull-down assay, with GST fusions to various parts of Id2 and either *in vitro* translated SAP-1-156 (including the ETS-domain, Figure 1A) or E47 (Figure 3B and C).

SAP-1 interacts strongly with the proteins possessing a HLH motif, wild-type Id2, Id2-ΔN and Id2-ΔC (Figure 3B, lanes 2, 5 and 6). Interaction is also seen with the Id2 HLH domain alone, albeit at a reduced level [Figure 3B, lane 7, ~15% wild-type (WT) protein]. However, very little interaction is seen with the double proline mutant Id2-HLH(pro) (Figure 3B, lane 8, <2% WT protein). Similarly, SAP-1 does not interact with either the N- or C-terminal regions of Id2 (Figure 3B, lanes 3 and 4). A known Id-binding protein, E47, was used as a positive control in these experiments and its binding to the Id2 deletions closely resembles that of SAP-1 (compare Figure 3B with 3C). Similar results were obtained using MyoD in place of E47 (data not shown). These results therefore indicate that the region of Id2 which interacts with SAP-1 is also required for binding to bHLH proteins such as E47 and MyoD.

Together, our data indicate that the HLH motif of the

Fig. 4. Ids disrupt ternary complex formation by Elk-1 and SAP-1. Gel retardation analysis of complex formation by TCFs in the presence of Id2. (A) Binary (2°) and ternary (3°) complex formation by full-length Elk-1 (lanes 1–2), SAP-1 (lanes 3–4), core^{SRF} and the *c-fos* SRE in the absence (lanes 1 and 3) or the presence (lanes 2 and 4) of *in vitro* translated Id2 protein. Id2 was added prior to the addition of the SRE to the binding reaction. (B) Ternary complex formation by full-length SAP-1 and the *c-fos* SRE in the absence of Id (lane 1) or the presence of equal molar amounts of *in vitro* translated Id1, Id2 and Id3 proteins (lanes 2–4). (C) Binary (2°) and ternary (3°) complex formation in the absence of TCFs (lanes 1–3) in presence of Elk1-168 (lanes 4–6) or SAP1-156 (lanes 7–9), core^{SRF} and the *c-fos* SRE. Increasing amounts (0 μ l, lanes 1, 4 and 7; 0.4 μ l, lanes 2, 5 and 8; 1.6 μ l, lanes 3, 6 and 9) of *in vitro* translated Id2 protein were added prior to the addition of SRE to the binding reaction. Three-fold (Elk1-168) and 6-fold (SAP1-156) reduction in ternary complex formation is observed at the highest concentrations of Id2 used. (D) As in (C), except that SRF was omitted from the reaction and the E74-binding site was used.

Id proteins is sufficient for binding to the TCFs, but maximal interaction requires the presence of sequences in either the N- or C-terminal regions.

Ids inhibit ternary complex formation by TCFs and SRF at the *c-fos* SRE

Ids bind to, and subsequently inhibit the DNA-binding activity of, bHLH proteins (Benezra *et al.*, 1990; Sun *et al.*, 1991; reviewed in Norton *et al.*, 1998). Similarly, as Id proteins bind to the TCFs, they may also affect the DNA-binding activity of TCFs. The ability of Id proteins to disrupt the formation of ternary TCF–SRF–SRE complexes was therefore tested.

Ternary complex formation by both Elk-1 (Figure 4A, lanes 1 and 2) and SAP-1 (Figure 4A, lanes 3 and 4) was inhibited by Id2. In contrast, the formation of binary SRF–SRE complexes was unaffected (Figure 4A and B, lanes

Fig. 5. Id2 actively disrupts complex formation by TCFs by acting on the ETS DNA-binding domain. (A) Gel-retardation analysis of binary (2°) and ternary (3°) complex formation by SAP1-156, core^{SRF} and the *c-fos* SRE in the presence of Id2. Ternary complex formation was allowed to proceed for 30 min prior to the addition of Id2. Samples were withdrawn at the indicated times following Id2 addition. (B) Sequences of the ets-binding motifs within the E74, CECI and SRE sites. Bases identified as important for TCF binding from a *c-fos* SRE in the presence of Id2. Ternary complex formation was Bases which differ from the optimal E74 motif are highlighted. (C) Gel-retardation analysis of binary (2°) complex formation by Elk1-93 and SAP1-92 on the CECI site. Increasing amounts (0 μ l, lanes 1 and 4; 0.4 μ l, lanes 2 and 5; 1.6 μ l, lanes 3 and 6) of Id2 were added prior to adding the CECI site to the reaction.

1–4, and Figure 4C, lanes 1–3). The relative ability of Id1, Id2 and Id3 to abrogate ternary complex formation was investigated (Figure 4B). Each of the Id proteins reduced ternary complex formation by full-length SAP-1. Id2 reduced complex formation to the greatest degree, followed by Id3 and then Id1. These results correlate with the strength of interaction detected using *in vitro* pull-down assays (Figure 2C).

The C-terminally truncated Elk-1 and SAP-1 proteins, Elk1-168 and SAP1-156 (Figure 1A), containing the ETS-domain and B-box regions, are sufficient for ternary complex formation (Dalton and Treisman, 1992; Janknecht and Nordheim, 1992) and interaction with Id proteins (Figure 1C). The Id proteins also inhibit ternary complex formation by Elk1-168 and SAP1-156 in a dose-dependent manner (Figure 4C, lanes 4–9) with 50% inhibition occurring at approximately a 1:1 stoichiometry (data not shown). The inhibitory activity of the Id proteins therefore acts on one of the two conserved N-terminal domains in the TCFs.

TCFs can also bind to high-affinity ets motifs in the absence of SRF (Janknecht *et al.*, 1994; Shore and Sharrocks, 1995). However, in contrast to their effect on ternary complex formation, the Ids do not affect the ability of Elk1-168 and SAP1-156 to bind to the high-affinity E74 site (Figure 4D).

Collectively, these data indicate that Id proteins inhibit ternary complex formation by interacting with the N-terminal part of the TCF protein.

Ids actively displace TCFs by inhibiting ETS-domain–DNA interactions

Ids can inhibit the formation of ternary TCF–SRF–SRE complexes (Figure 4). The ability of Ids to displace TCFs from preformed ternary complexes was investigated. Following ternary complex formation by SAP1-156, Id2 was added and samples tested (Figure 5A). Rapid decay

Fig. 6. *c-fos* and *Id1–3* expression show a temporal overlap following serum induction. NIH 3T3 cells were serum starved for 24 h, then stimulated with 15% fetal calf serum (FCS) and harvested at the times indicated. Northern blots are shown after probing for each *Id* gene and for *c-fos*. RNA loadings were normalized by reference to the 28S rRNA, visible on an ethidium bromide-stained gel (shown in the bottom panel).

of the ternary complex was observed, indicating that Ids can actively disrupt ternary complexes (Figure 5A, lanes 1–6).

Both protein–DNA and protein–protein interactions are essential for ternary complex formation by TCFs (Dalton and Treisman, 1992; Janknecht and Nordheim, 1992; Shore and Sharrocks, 1994). Ids might therefore act to disrupt either of these two types of intermolecular interactions. Binding of TCFs to the high-affinity E74 motif was unaffected by Ids (Figure 4D). However, the ets motif within the *c-fos* SRE represents a lower affinity TCF-binding motif (Shore and Sharrocks, 1995). We therefore tested whether Id2 could disrupt the binding of TCFs to lower affinity ets motifs such as in the CECI site (Ling *et al.*, 1998). The binding of the ETS-domains from Elk-1 and SAP-1 to this site were both inhibited in a dose-dependent manner by Id2 (Figure 5C, lanes 1–6). Consistent with the observation that Ids interact with the ETS-domain (Figure 1C), Id2 inhibits DNA binding by this isolated TCF domain (Figure 4C).

Together these results indicate that Ids can actively displace preformed ternary complexes and that this inhibition occurs, at least in part, by affecting DNA binding by the ETS-domain on suboptimal ets motifs.

***c-fos* and *Id1–3* expression show a temporal overlap following serum induction**

c-fos and *Id1–3* represent immediate-early genes (Christy *et al.*, 1991; Deed *et al.*, 1993; Barone *et al.*, 1994; Hara *et al.*, 1994; reviewed in Herschman, 1991; Murphy *et al.*, 1997) that are activated following stimulation with serum and other mitogens. In order to determine the relative temporal expression profiles of these genes in NIH 3T3 cells following serum stimulation, we analysed their expression using Northern blotting (Figure 6). The expression of *c-fos* peaked 30 min after serum stimulation, was

Fig. 7. Id2 attenuates ternary-complex-mediated reporter activity at the *c-fos* SRE. The layout of the reporters is represented as diagrammatic inserts. (A) Id2 down-regulates a *c-fos* promoter-regulated CAT reporter activated by the MEK/ERK pathway. NIH 3T3 cells were co-transfected with 1 µg of -711fosCAT reporter vector, 0.5 µg pCMVMEK-1(DN) either in the absence or in the presence of 0.5, 1.0, 3.0 and 6.0 µg of pcDNAId2, respectively. (B) Elk-1 can rescue the down-regulatory effect of Id2. NIH 3T3 cells were co-transfected with 1 µg of -711fosCAT reporter vector, 0.5 µg pCMVMEK-1(CA) and 4.0 µg of pcDNAId2 either in the absence or in presence of 0.5 or 2.5 µg of pCMVElk-1. (C) The SRE is sufficient to mediate the Id down-regulation of the *fos* promoter. NIH 3T3 cells were co-transfected with 1 µg of SREx2-luc reporter vector, 0.5 µg pCMVMEK-1(DN) either in the absence or presence of 0.5, 1.0, 3.0 and 6.0 µg of pcDNAId2, respectively. Data are presented relative to the reporter plasmid alone (taken as 1). All values and standard errors were calculated from averages of triplicate samples and are representative of two or three independent experiments.

maintained at a high level for 60 min and then gradually returned to basal levels after 2.5 h. *Id1*, *Id2* and *Id3* were induced between 30 and 60 min after serum stimulation and their expression was maintained for at least 4 h, consistent with previous studies (Barone *et al.*, 1994; Hara *et al.*, 1994). In contrast, *Id4* was not detectably expressed in this cell type (data not shown).

Thus, significant overlap in the expression profile of the *Ids* and *c-fos* is observed, with the expression of the *Ids* coinciding with the shut off of the *c-fos* promoter.

Ids inhibit *c-fos* promoter activation via the SRE

The TCFs play a pivotal role in *c-fos* induction following growth factor and mitogenic stimulation, and the Id proteins interact and inhibit ternary complex formation by the TCFs. The temporal overlap in *Id* and *c-fos* expression suggests that the Id proteins might participate in the down-regulation of *c-fos* and other immediate-early genes. To test this hypothesis, we examined the effect of overexpression of Id2 on the activity of a *c-fos* promoter-driven reporter construct (Figure 7A). A constitutively active

MEK protein was co-transfected to stimulate the *c-fos* reporter via the extracellular signal-related kinase (ERK) pathway (Yang *et al.*, 1998a). Co-transfection of increasing quantities of Id2 caused a dose-dependent reduction in the activity of the reporter in response to MEK induction. At the highest level of transfected Id2, the reporter was inhibited to below basal levels (Figure 7A). ERK-mediated induction of the *c-fos* promoter is thought to take place mainly via the TCF component of the ternary TCF–SRF–SRE complex (reviewed in Treisman, 1994). If Id proteins function by interacting with Elk-1 and disrupting this ternary complex, then overexpression of Elk-1 should rescue the activity of the Id-inhibited *c-fos* promoter. Co-transfection of Id2 reduced MEK-inducible activation of the *c-fos* promoter. However, co-transfection of increasing quantities of Elk-1 caused a dose-dependent rescue of *c-fos* reporter gene activity (Figure 7B), indicating that inhibition of the *c-fos* promoter by the Ids possibly involves interaction with the TCFs bound at the SRE.

In order to determine whether the Id proteins cause *c-fos* promoter inhibition through the SRE, the ability of Id2 to inhibit the activity of an SRE-driven reporter construct was analysed. Co-transfection of MEK enhances the activity of the SRE-luc reporter. However, co-transfection of increasing quantities of Id2, causes a dose-dependent reduction in the activity of the reporter in response to MEK induction. As observed on the intact *c-fos* promoter, at the highest level of transfected Id2, the SRE-driven reporter was inhibited to below basal levels (Figure 7C).

Together, these results demonstrate that the Id proteins can act to inhibit MEK-inducible *c-fos* promoter activation. This inhibition occurs via the TCF component of the ternary complex formed on the *c-fos* SRE.

Discussion

The TCF subfamily of ETS-domain transcription factors plays a pivotal role in regulating the expression of immediate-early genes such as *c-fos* and *egr-1* in response to a variety of extracellular signals including various mitogens and growth factors (reviewed in Cahill *et al.*, 1996). These signals are transduced to the TCFs by the ERK MAPK pathway (reviewed in Treisman, 1994). Genes encoding the Id proteins are also activated in an immediate-early fashion following mitogenic stimulation. Here, we demonstrate that following serum induction, the expression of *Id1–3* in fibroblasts is activated shortly after *c-fos* and is maintained as *c-fos* expression is subsequently down-regulated, suggesting a possible role for the Ids in this down-regulation process. Indeed, overexpression of Id2 causes a reduction in the activity of a *c-fos* promoter-driven reporter construct. This inhibition occurs via the SRE and can be rescued by Elk-1 overexpression, indicating that the ternary TCF–SRF–SRE complex is the target for Id-mediated inhibition of *c-fos* promoter activity. Consistent with this hypothesis, we show that the Id proteins bind to and inhibit DNA binding by the TCF component of this ternary complex. Collectively, our results reveal a novel facet of Id protein function in the coordination of early responses to mitogenic signalling.

Mechanism of Id-mediated inhibition of DNA binding by TCFs

Interactions between the Id proteins and the TCFs are mediated by the ETS-domain of the TCF component and

the HLH motif in the Ids. In the Ids, additional N- and C-terminal residues apparently enhance these interactions, most probably by stabilizing the structure of the HLH motif. This interpretation is consistent with the recent observation that regions from outside the HLH motif are required for the stability of the HLH motif in the E-class of bHLH proteins (Goldfarb *et al.*, 1998). Id2 interacts preferentially with and inhibits DNA binding by SAP-1 and SAP-2, which is consistent with the greater similarity shared between these two proteins when compared with Elk-1. Interactions between Id proteins and ETS-domain proteins from other subfamilies, including PEA3 and Fli-1, can also be detected (P.R.Yates and A.D.Sharrock, unpublished data), which is consistent with the role of the highly conserved ETS-domain in this interaction. However, in the case of the non-TCF ETS-domain proteins, the functional consequences of these interactions are currently unclear. Interestingly, the HLH motif in the Id proteins exhibits sequence similarity with the NID domain in SAP-2 which acts *in cis* to inhibit DNA binding by SAP-2 (Maira *et al.*, 1996). This raises the possibility that the Id proteins inhibit DNA binding in an analogous manner to the NID. Inhibition of DNA binding by Id proteins takes place in a site-dependent manner. The binding of TCFs to low-affinity ets motifs (such as in the *c-fos* SRE), either alone or in ternary complexes with SRF, is inhibited by Ids but binding to high-affinity ets motifs (such as the E74 site) is unaffected. Ids can inhibit DNA binding by TCFs when added before or after the formation of TCF–DNA complexes, suggesting that they are able to disrupt preformed ternary complexes. This also suggests that the mechanism of inhibition of DNA binding possibly involves an allosteric effect rather than simple blocking of the DNA-binding surface. Indeed, the observation that Ids are incapable of blocking the binding of the TCFs to the high-affinity E74 site supports this hypothesis. A model has been proposed previously in which residues in SAP-1 can act as allosteric effectors which alter DNA contacts made by other residues and hence DNA-binding specificity, but do not affect binding to high-affinity sites (Shore *et al.*, 1996; Mo *et al.*, 1998). The binding of Id proteins may promote similar allosteric changes which only affect binding to lower affinity sites.

Id1, Id2 and Id3 can all interact with and inhibit DNA binding by the TCFs. However, Id1 binds and inhibits with a lower efficiency, suggesting some specificity in their interaction. Id proteins have previously been shown to inhibit DNA binding by bHLH proteins (Benezra *et al.*, 1990; Sun *et al.*, 1991; reviewed in Norton *et al.*, 1998) by forming inactive heterodimers. However, little sequence similarity exists between the ETS-domain and bHLH proteins, indicating that the mechanisms of inhibiting DNA binding possibly differ. Id proteins do not, however, act as pleiotropic inhibitors of DNA binding as they do not affect DNA binding by the MADS-box proteins SRF (Figure 4) and MEF2A (E.C.Roberts and A.D.Sharrock, unpublished data).

The role of Ids in regulating immediate-early gene expression

Following mitogenic stimulation, the *Id* genes are expressed in a classic immediate-early manner (Christy *et al.*, 1991; Deed *et al.*, 1993; Barone *et al.*, 1994; Hara

et al., 1994). However, their expression is delayed in comparison with other immediate-early genes such as *c-fos* (Figure 6). It has previously been demonstrated that the product of another immediate-early gene (*egr-1*) acts to up-regulate the expression of *Id1* (Tournay and Benezra, 1996) and probably also *Id3* (Christy *et al.*, 1991), suggesting the existence of a cascade of genes which are sequentially activated following serum stimulation. In this study, we demonstrate that the Id proteins can inhibit the *c-fos* promoter, suggesting the existence of a negative feedback loop which acts to down-regulate genes such as *c-fos* and *egr-1*. Consistent with this hypothesis is the observation that the Ids elicit their function by affecting the activity of the TCFs which have been implicated in regulating both of these genes (reviewed in Treisman, 1994; Watson *et al.*, 1997). Activation of the TCFs is mediated by phosphorylation of their transcriptional activation domains by MAPKs and leads to enhancement of both their DNA binding and transcriptional activation activities (reviewed in Treisman, 1994). However, the occupancy of the ets motif within the *c-fos* SRE does not change following growth factor stimulation (Herrera *et al.*, 1989). An exchange of active and inactive TCF molecules has been suggested to occur with the phosphorylated form displacing the non-phosphorylated form (Gille *et al.*, 1992). Alternatively, the TCFs could be phosphorylated while bound to the promoter (reviewed in Treisman, 1994). Recently, it has been demonstrated that the major constituent of ternary TCF–SRF–SRE complexes in several cell types is Net-b, a truncated version of the murine SAP-2 homologue Net (Giovane *et al.*, 1997). Net-b lacks the transcriptional activation domain and acts as a dominant-negative inhibitor of SRE function. Thus, one role of the Id proteins in this context would be to promote dissociation of the phosphorylated TCFs and allow their replacement with either inactive or dominant-negative forms of the TCFs, thereby providing an elegant mechanism for down-regulating and resetting the promoter.

In summary, we have demonstrated direct functional interactions between the Id HLH proteins and members of the TCF subfamily of ETS-domain transcription factors. These observations increase the repertoire of proteins known to be inhibited by Id proteins. In addition, the discovery that TCFs interact with Id proteins contributes to our understanding of the intricate mechanisms by which these transcription factors are regulated. Together, our results contribute to the understanding of the early events that follow mitogenic signalling and progression through the G₁ phase of the cell cycle and how they are controlled in an integrated manner.

Materials and methods

Plasmid constructs

The following plasmids were used to express C-terminal hexa histidine-tagged proteins in *Escherichia coli*. pAS413 encodes full-length Elk-1 (amino acids 1–428) (Ling *et al.*, 1997). pAS275 encodes full-length SAP-1 (amino acids 1–431) (Shore *et al.*, 1996).

The following plasmids were used to express GST-fusion proteins in *E. coli*. pAS58 (encoding core^{SRF} amino acids 132–222), pGEX–MyoD [encoding GST–MyoD; full-length MyoD (amino acids 1–318) fused to GST; kindly provided by H. Weintraub] and pGNElk (encoding GST–Elk-1-205; Elk-1 amino acids 1–205 fused to GST) (Gille *et al.*, 1995a) have been described previously. pGEX–Id2 encodes GST–Id2 (Id2 amino acids 1–134 fused to GST) (Deed *et al.*, 1997). GST–Id2 deletion

constructs, pAS910, pAS911, pAS912, pAS913, pAS914, encoding amino acids 1–36, 76–134, 36–134, 1–76, 36–76 of Id2 fused to GST, respectively, were constructed by PCR amplification from pcDNA3Id2 using oligonucleotide primer pairs ADS630–ADS612, ADS614–ADS631, ADS606–ADS631, ADS630–ADS613, ADS606–ADS613, respectively, and ligated as *Bam*HI–*Xho*I fragments into the same sites of pGEX-KG (Guan and Dixon, 1991). pAS915 (GST–Id2 deletion construct containing HLH domain amino acids 36–76 with the point mutations S44P and L68P) was constructed by PCR amplification from pcDNAId2H1H2br using the oligonucleotide pair ADS606–ADS613, followed by ligation as a *Bam*HI–*Xho*I fragment into the same sites of pGEX-KG. pAS535 (encoding GST–SAP-1-298; SAP-1 amino acids 1–298 fused to GST) was constructed by ligating an *Nco*I–*Sac*I fragment into the same sites of pGEX-KG.

The following plasmids were used in mammalian cell transfections. pF711CAT contains human *c-fos* promoter –711 to +42 ahead of the CAT gene (Treisman, 1985). pSRE-luc contains two copies of the *c-fos* serum response element (nucleotides –357 to 275) upstream from a minimal tk promoter and the luciferase gene (Seth *et al.*, 1992). The expression vector for constitutively active MEK-1(ΔN S218E–S222D) (pCMV–MEK–DN) was provided by N. Ahn (Mansour *et al.*, 1994). pAS383 encodes full-length Flag-epitope-tagged Elk-1 driven by a CMV promoter (Yang *et al.*, 1998a).

The following plasmids were used for *in vitro* transcription/translation and/or transfection purposes. pcDNA3Id1 (encoding full-length Id1; amino acids 1–148), pcDNA3Id2 (encoding full-length Id2; amino acids 1–134), pcDNA3Id3 (encoding full-length Id3; amino acids 1–119) (Norton and Atherton, 1998), pAS728 (encoding full-length Elk-1; amino acids 1–428) (Ling *et al.*, 1997), pAS96 (encoding Elk-1-168; amino acids 1–168) and pAS72 (encoding Elk-1-93; amino acids 1–93) (Shore and Sharrocks, 1994), pAS136 (encoding SAP-1-92; amino acids 1–92), pAS168 (encoding SAP-1-156; amino acids 1–156) (Shore and Sharrocks, 1995), pT7.SAP-1 (encoding full-length SAP-1; amino acids 1–431) (Dalton and Treisman, 1992) and pT7.SAP-2 (encoding full-length SAP-2; amino acids 1–407) (Price *et al.*, 1995) have been described previously. pAS700 (encoding Elk-1-306; amino acids 1–306) was constructed by ligating the *Nco*I–*Bgl*III fragment from pAS413 into the *Nco*I–*Bam*HI-cleaved pAS37 (Sharrocks *et al.*, 1993a). A full-length cDNA encoding E47 (Sun *et al.*, 1991) was cloned into pcDNA3 for *in vitro* transcription/translation.

Protein expression

The synthesis of proteins by *in vitro* transcription and translation was carried out with the TNT-coupled reticulocyte lysate system (Promega, WI) according to the manufacturer's recommendations. Newly synthesized ³⁵S-labelled proteins were analysed by SDS–PAGE followed by visualization and quantification by phosphorimager analysis (Fuji BAS-1500 phosphorimager and TINA 2.08e software). GST fusion proteins (Shore and Sharrocks, 1994) and hexahistidine-tagged proteins (Yang *et al.*, 1998a) were purified as described previously. The purity of proteins was determined by SDS–PAGE and the concentration by Coomassie Blue protein assay reagent (Pierce, IL).

In vitro protein–protein interaction assays

Interactions between GST–Elk-1-205 and GST–SAP-1-298 and *in vitro* translated Ids and between GST–Id2 and truncated derivatives of GST–Id2 and *in vitro* translated full-length and truncated derivatives of Elk-1 and SAP-1 were investigated using pull-down assays as described previously (Shore and Sharrocks, 1994).

Gel-retardation assays

Gel-retardation assays were performed with ³²P-labelled probes as described previously (Sharrocks *et al.*, 1993b). The binding sites include the *c-fos* SRE, the E74 site (Shore and Sharrocks, 1994) and the CECI site (Ling *et al.*, 1998). The CECI site is an artificial DNA-binding site which contains a low-affinity SRF binding site and an ets motif corresponding to the S30 site identified from a DNA-binding site selection study (Shore and Sharrocks, 1995). DNA–protein complexes were formed at room temperature for 15 min using bacterially expressed core^{SRF} and bacterially expressed or *in vitro*-translated TCF derivatives. *In vitro* translated Id proteins were either added at the same time as the TCFs or after the end of this incubation period. When Ids were added following pre-incubations, reactions were allowed to proceed at room temperature for a further 15 min. Generally, reactions were normalized for reticulocyte lysate content. Protein–DNA complexes were analysed on non-denaturing 5% polyacrylamide gels cast in 0.5× Tris–borate–EDTA and visualized by autoradiography and phosphorimaging.

Cell culture transfection and reporter gene assays

NIH 3T3 and Cos-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, MD) and 25 mM glucose. Triplicate transfection experiments were carried out in six-well plates using Superfect reagent according to the manufacturer's recommendations (Qiagen, Hilden, Germany). For reporter gene assays, *c-fos*-promoter-driven or SRE-driven reporters were co-transfected alongside vectors encoding constitutively active MEK-1, Id2 and Elk-1. DNA concentrations were normalized with pcDNA3 vector.

Transfected cells were incubated in Optimem (Gibco-BRL, MD) for 24 h after transfection and lysed in 200 µl lysis buffer [100 mM K₂PO₄, pH 7.8, 0.2% Triton X-100, 0.5 mM dithiothreitol (DTT)]. Cell debris was removed by centrifugation at 14 000 g for 15 min at 4°C and the supernatant assayed for CAT activity using a TLC assay as described previously (Ling *et al.*, 1997). Luciferase assays were carried out as described previously (Yang *et al.*, 1998). Data were quantified by phosphorimager analysis and the data presented graphically using Microsoft Excel software. Transfection efficiency was monitored by measuring the β-galactosidase activity from co-transfected pCH110 plasmid (0.5 µg) (Pharmacia KB Biotechnology Inc., Uppsala, Sweden). β-galactosidase activities were determined by the use of a chemiluminescent substrate in the Galacto-light Plus kit (tropix, MA).

Immunoprecipitation

The antibody matrix was prepared by covalently coupling an Id2-specific rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., CA) to protein A beads (Gersten and Marchionis, 1978). Cos-7 cell extracts containing overexpressed Id2 and Flag-tagged Elk-1 proteins were prepared from two 35-mm dishes in 400 µl of Triton lysis buffer (20 mM Tris pH 7.4, 137 mM sodium chloride, 25 mM β-glycerophosphate, 50 mM sodium fluoride, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 2 mM sodium pyrophosphate, 10 mM MgCl₂) containing protease inhibitors (at final concentrations: leupeptin 2 µg/ml, pepstatin A 1 µg/ml, PMSF 100 µg/ml and aprotinin 2 µg/ml). Antibody matrix (25 µl) was incubated with the cell extract with rotation for 4 h at 4°C. Complexes were washed three times with Triton lysis buffer (TLB) and subjected to SDS-PAGE followed by Western blot analysis. Immunoprecipitated Elk-1 was detected by immunoblot analysis with a mouse monoclonal anti-M2 Flag antibody (Kodak, NY). Immune complexes were detected using horseradish-peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence (Amersham, Amersham, UK).

Northern hybridization

Total cellular RNA was prepared by using an RNeasy extraction kit (Qiagen, Crawley, UK) and 10 µg samples were electrophoresed on 1.2% agarose-2.2 M formaldehyde gels. After staining with ethidium bromide, gels were blotted onto GeneScreen Plus nylon membranes (NEN-Dupont, MA) and hybridized with random-primed ³²P-labelled probes essentially as described previously (Deed *et al.*, 1993).

Figure generation

Figures were generated electronically from scanned images of autoradiographic images by using Picture Publisher (Micrografix) and Powerpoint 7.0 (Microsoft) software. Results are representative of the original autoradiographic images.

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References

Barone,M.V., Pepperkok,R., Peverali,F.A. and Philipson,L. (1994) Id proteins control growth induction in mammalian cells. *Proc. Natl Acad. Sci. USA*, **91**, 4985–4988.

Benezra,R., Davis,R.L., Lockshon,D., Turner,D.L. and Weintraub,H.

(1990) The protein Id: a negative regulator of helix–loop–helix DNA binding proteins. *Cell*, **61**, 49–59.

Biggs,J.R., Murphy,E.V. and Israel,M.A. (1992) A human Id-like HLH protein expressed during early development. *Proc. Natl Acad. Sci. USA*, **89**, 1512–1516.

Cahill,M.A., Janknecht,R. and Nordheim,A. (1996) Signalling pathways: Jack of all cascades. *Curr. Biol.*, **6**, 16–19.

Christy,B.A., Sanders,L.K., Lau,L.F., Copeland,N.G., Jenkins,N.A. and Nathans,D. (1991) An Id-related helix–loop–helix protein encoded by a growth factor-inducible gene. *Proc. Natl Acad. Sci. USA*, **88**, 1815–1819.

Dalton,S. and Treisman,R. (1992) Characterisation of SAP-1, a protein recruited by serum response factor to the *c-fos* serum response element. *Cell*, **68**, 597–612.

Dang,W., Sun,X.-H. and Sen,R. (1998) ETS-mediated cooperation between basic helix–loop–helix motifs of the immunoglobulin µ heavy-chain gene enhancer. *Mol. Cell. Biol.*, **18**, 1477–1488.

Deed,R.W., Bianchi,S.M., Atherton,G.T., Johnston,D., Santibanez-Akaref,M., Murphy,J.J. and Norton,J.D. (1993) An immediate early gene encodes an Id-like helix–loop–helix protein and is regulated by protein kinase C activation in diverse cell types. *Oncogene*, **8**, 599–607.

Deed,R.W., Hara,E., Atherton,G.T., Peters,G. and Norton,J.D. (1997) Regulation of Id3 cell cycle function by Cdk-2-dependent phosphorylation. *Mol. Cell. Biol.*, **17**, 6815–6821.

Florio,M., Hernandez,M., Yang,H., Shu,H.-K., Cleveland,J.L. and Israel,M.A. (1998) Id2 promotes apoptosis by a novel mechanism independent of dimerisation to basic helix–loop–helix factors. *Mol. Cell. Biol.*, **18**, 5435–5444.

Gersten,D.M. and Marchionis,J.J. (1978) A rapid and novel method for the solid-phase derivatization of IgG antibodies for immune-affinity chromatography. *J. Immunol. Methods*, **24**, 305–309.

Gille,H., Sharrocks,A.D. and Shaw,P.E. (1992) Phosphorylation of transcription factor p62^{TCF} by MAP kinase stimulates ternary complex formation at the *c-fos* promoter. *Nature*, **358**, 415–417.

Gille,H., Kortenjann,M., Thomae,O., Moomaw,C., Slaughter,C., Cobb,M.H. and Shaw,P.E. (1995a) ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. *EMBO J.*, **14**, 951–962.

Gille,H., Strahl,T. and Shaw,P.E. (1995b) Activation of ternary complex factor Elk-1 by stress-activated protein kinases. *Curr. Biol.*, **5**, 1191–1200.

Giovane,A., Pintzas,A., Maira,S.-M., Sobieszcuk,P. and Wasyluk,B. (1994) Net, a new *ets* transcription factor that is activated by Ras. *Genes Dev.*, **8**, 1502–1513.

Giovane,A., Sobieszcuk,P., Ayadi,A., Maira,S.-M. and Wasyluk,B. (1997) Net-b, a Ras-insensitive factor that forms ternary complexes with the serum response element of the *fos* promoter. *Mol. Cell. Biol.*, **17**, 5667–5678.

Goldfarb,A.N., Lewandowska,K. and Pennell,C.A. (1998) Identification of a highly conserved module in E proteins required for *in vivo* helix–loop–helix dimerization. *J. Biol. Chem.*, **273**, 2866–2873.

Guan,K. and Dixon,J.E. (1991) Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.*, **192**, 262–267.

Hara,E., Yamaguchi,T., Nojima,H., Ide,T., Campisi,J., Okayama,H. and Oda,K. (1994) Id-related genes encoding HLH proteins are required for G₁ progression and are repressed in senescent human fibroblasts. *J. Biol. Chem.*, **269**, 2139–2145.

Herrera,R.E., Shaw,P.E. and Nordheim,A. (1989) Occupation of the *c-fos* serum response element *in vivo* by a multi-protein complex is unaltered by growth factor induction. *Nature*, **340**, 68–70.

Herschmann,H.R. (1991) Primary response genes induced by growth factors and tumour promoters. *Annu. Rev. Biochem.*, **60**, 281–319.

Iavarone,A., Garg,P., Lasorella,A., Hsu,J. and Israel,M.A. (1994) The helix–loop–helix protein Id-2 enhances cell proliferation and binds to the retinoblastoma protein. *Genes Dev.*, **8**, 1270–1284.

Janknecht,R. and Nordheim,A. (1992) Elk-1 protein domains required for direct and SRF-assisted DNA-binding. *Nucleic Acids Res.*, **20**, 3317–3324.

Janknecht,R., Zinck,R., Ernst,W.H. and Nordheim,A. (1994) Functional dissection of the transcription factor Elk-1. *Oncogene*, **9**, 1273–1278.

Ling,Y., Lakey,J., Roberts,E.C. and Sharrocks,A.D. (1997) Molecular characterisation of the B-box protein–protein interaction motif of the ETS-domain transcription factors Elk-1. *EMBO J.*, **16**, 2431–2440.

Ling,Y., West,A.G., Roberts,E.C., Lakey,J.H. and Sharrocks,A.D. (1998) Interaction of transcription factors with SRF: identification of the Elk-1 binding surface. *J. Biol. Chem.*, **273**, 10506–10514.

- Lopez,M., Oettgen,P., Akbarali,Y., Dendorfer,U. and Liberman,T.A. (1994) ERP, a new member of the *ets* transcription factor/oncoprotein family: cloning, characterization and differential expression during B-lymphocyte development. *Mol. Cell. Biol.*, **14**, 3292–3309.
- Maira,S.-M., Wurtz,J.-M. and Wasylyk,B. (1996) Net (ERP/SAP2), one of the Ras-inducible TCFs, has a novel inhibitory domain with resemblance to the helix–loop–helix motif. *EMBO J.*, **15**, 5849–5865.
- Mansour,S.J., Matten,W.T., Hermann,A.S., Candia,J.M., Rong,S., Fukasawa,K., Vande-Woude,G.F. and Ahn,N.G. (1994) Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science*, **265**, 966–970.
- Marais,R., Wynne,J. and Treisman,R. (1993) The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell*, **73**, 381–393.
- Mo,Y., Vaessen,B., Johnston,K. and Marmorestein,R. (1998) Structures of SAP-1 bound to DNA sequences from the E74 and c-fos promoters provide insights into how ETS proteins discriminate between related DNA targets. *Mol. Cell*, **2**, 201–212.
- Murphy,J.J., Newton,J.S. and Norton,J.D. (1997) Early response genes and B cell activation. In Harnett,M.M. and Righley,K.P. (eds), *Lymphocyte Signalling: Mechanisms, Subversion and Manipulation*. John Wiley and Sons Ltd, New York, NY, pp. 183–202.
- Norton,J.D. and Atherton,G.T. (1998) Coupling of cell growth control and apoptosis functions of Id proteins. *Mol. Cell. Biol.*, **18**, 2371–2381.
- Norton,J.D., Deed,R.W., Craggs,G. and Sablitzky,F. (1998) Id helix–loop–helix proteins in cell growth and differentiation. *Trends Cell Biol.*, **8**, 58–65.
- Price,M.A., Rogers,A.E. and Treisman,R. (1995) Comparative analysis of the ternary complex factors Elk-1, SAP-1a and SAP-2 (ERP/NET). *EMBO J.*, **14**, 2589–2601.
- Rao,V.N., Huebner,K., Isobe,M., Ab-Rushidi,A., Croce,C.M. and Reddy,E.S.P. (1989) Elk, tissue-specific *ets*-related genes on chromosomes X and 14 near translocation breakpoints. *Science*, **244**, 66–70.
- Riechmann,V., van Cruchten,I. and Sablitzky,F. (1994) The expression pattern of Id4, a novel dominant negative helix–loop–helix protein, is distinct from Id1, Id2 and Id3. *Nucleic Acids Res.*, **22**, 749–755.
- Seth,A., Gonzalez,F.A., Gupta,S., Raden,D.L. and Davis,R.J. (1992) Signal transduction within the nucleus by mitogen-activated protein kinase. *J. Biol. Chem.*, **267**, 24796–24804.
- Sharrocks,A.D., von Hesler,F. and Shaw,P.E. (1993a) The identification of elements determining the different DNA-binding specificities of the MADS box proteins p67SRF and RSRFC4. *Nucleic Acids Res.*, **21**, 215–221.
- Sharrocks,A.D., Gille,H. and Shaw,P.E. (1993b) Identification of the amino acids essential for DNA binding and dimerization in p67^{SRF}: implications for a novel DNA-binding motif. *Mol. Cell. Biol.*, **13**, 123–132.
- Shoji,W., Inoue,T., Yamamoto,T. and Obinata,M. (1995) MIDA1, a protein associated with Id, regulates cell growth. *J. Biol. Chem.*, **270**, 24818–24825.
- Shore,P. and Sharrocks,A.D. (1994) The transcription factors Elk-1 and serum response factor interact by direct protein–protein contacts mediated by a short region of Elk-1. *Mol. Cell. Biol.*, **14**, 3283–3291.
- Shore,P. and Sharrocks,A.D. (1995) The ETS-domain transcription factors Elk-1 and SAP-1 exhibit differential DNA binding specificities. *Nucleic Acids Res.*, **23**, 4698–4706.
- Shore,P., Whitmarsh,A.J., Bhaskaran,R., Waltho,J., Davis,R.J. and Sharrocks,A.D. (1996) Determinants of DNA binding specificity of ETS-domain transcription factors. *Mol. Cell. Biol.*, **16**, 3338–3349.
- Sieweke,M.H., Tekotte,H., Jarosch,U. and Graf,T. (1998) Cooperative interaction of ETS-1 with USF-1 required for HIV-1 enhancer activity in T cells. *EMBO J.*, **17**, 1728–1739.
- Sun,X.-H., Copeland,N.G., Jenkins,N.A. and Baltimore,D. (1991) Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix–loop–helix proteins. *Mol. Cell. Biol.*, **11**, 5603–5611.
- Tournay,O. and Benezra,R. (1996) Transcription of the dominant-negative helix–loop–helix protein Id1 is regulated by a protein complex containing the immediate-early gene *Egr-1*. *Mol. Cell. Biol.*, **16**, 2418–2430.
- Treisman,R. (1985) Transient accumulation of c-fos RNA following serum stimulation requires a conserved 5' element and c-fos 3' sequences. *Cell*, **42**, 889–902.
- Treisman,R. (1994) Ternary complex factors: growth regulated transcriptional activators. *Curr. Opin. Gen. Dev.*, **4**, 96–101.
- Watson,D.K., Robinson,L., Hodge,D.R., Kola,I., Papas,T.K. and Seth,A. (1997) FLI1 and EWS-FLI1 function as ternary complex factors and ELK1 and SAP1a function as ternary and quaternary complex factors on the Egr1 promoter serum response elements. *Oncogene*, **14**, 213–221.
- Yang,S.-H., Whitmarsh,A.J., Davis,R.J. and Sharrocks,A.D. (1998a) The Elk-1ETS-domain transcription factor contains a MAP kinase targeting motif. *Mol. Cell. Biol.*, **18**, 710–720.
- Yang,S.-H., Whitmarsh,A.J., Davis,R.J. and Sharrocks,A.D. (1998b) Differential targeting of MAP kinases to the ETS-domain transcription factor Elk-1. *EMBO J.*, **17**, 1740–1749.

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