Identification of Amino Acid Residues in the ETS Transcription Factor Erg That Mediate Erg-Jun/Fos-DNA Ternary Complex Formation*

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Jun, Fos, and Ets proteins belong to distinct families of transcription factors that target specific DNA elements often found jointly in gene promoters. Physical and functional interactions between these families play important roles in modulating gene expression. Previous studies have demonstrated a direct interaction between the DNA-binding domains of the two partners. However, the molecular details of the interactions have not been investigated so far. Here we used the known three-dimensional structures of the ETS DNA-binding domain and Jun/Fos heterodimer to model an ETS-Jun/Fos-DNA ternary complex. Docking procedures suggested that certain ETS domain residues in the DNA recognition helix α9 interact with the N-terminal basic domain of Jun. To support the model, different Erg ETS domain mutants were obtained by deletion or by single amino acid substitutions and were tested for their ability to mediate DNA binding, Erg-Jun/Fos complex formation, and transcriptional activation. We identified point mutations that affect both the DNA binding properties of Erg and its physical interaction with Jun (R367K), as well as mutations that essentially prevent transcriptional synergy with the Jun/Fos heterodimer (Y371V). These results provide a framework of the ETS/bZIP interaction linked to the manifestation of functional activity in gene regulation.

In eukaryotes, gene expression appears to be regulated by the assembly of various combinations of transcription factors at promoters and enhancers. The ability of transcription factors to interact specifically with one another, resulting in the formation of hetero-oligomeric complexes, enables the generation of diverse inducible and developmentally regulated programs of gene expression. Biochemical and functional characterization of transcription factor complexes has shown that the structural information necessary for their assembly is provided by both protein-protein and protein-DNA contacts (1). Particularly, Ets and Jun/Fos family members commonly function as part of the integrated regulatory complexes. For example, complex formation of the Ets protein Elk-1/SAP-1 with the serum responsive

factor is critical for the regulation of the c-fos promoter (2); the Ets-related protein GABPα forms heterotetramers with GABPβ to activate the immediate early promoters of HSV-1 (3–5); the association of Pu-1 with NF-EM5/Pip is important for the regulation of immunoglobulin light chain enhancers (6, 7); and the binding of a Jun/Fos heterodimer along with the nuclear factor of activated T cells to composite elements is critical for the regulation of genes involved in T cell activation (8). The Ets family proteins share a highly conserved 85-amino acid winged helix-turn-helix DNA-binding domain (ETS domain) that is able to bind the consensus DNA core sequence 5′-GGA(AT)3′ and to engage in protein-protein interactions (9–11). The Jun and Fos families consist of related bZIP1 proteins that are able to heterodimerize and bind the consensus DNA sequence 5′-TGACTCA-3′ (12). Ets proteins act synergistically with a variety of other transcription factors to regulate many cellular and viral promoters and enhancers (9, 10, 13). The Jun/Fos heterodimer is one of the well characterized partners of Ets proteins (10, 13). In vitro binding studies have demonstrated a direct interaction of various Ets proteins like Ets-1, Elf-1, Pu-1, Fli-1, Ets-2, and Erg with the Jun protein moiety but never with Fos alone (14–17). These direct protein interactions have been shown to involve the DNA-binding domain of the two partners. However, the amino acid residues critical for these highly intricate interactions have not been precisely mapped so far.

Here we studied the molecular contacts between the human Erg protein and the Jun/Fos heterodimer to gain insights into the general mechanisms by which these two families of transcription factors interact to regulate gene expression. In this respect, we used molecular modeling techniques and the available crystal structures of the ETS domain and the Jun/Fos heterodimer complexed to DNA to predict individual residues involved in the Ets-Jun/Fos-DNA ternary complex formation. The selected ETS domain residues were thus mutated and then tested for their ability to support Jun/Fos recruitment in vitro and in vivo. Interestingly two conserved amino acids in the ETS domain (residues Arg367 and Tyr371 in Erg) are required for efficient recruitment of the Jun/Fos heterodimer. Whereas the R367K substitution abolished DNA binding, interaction with Jun, and consequently transcriptional cooperation, the Y371V mutation abrogated interaction with Jun and synergy without abolishing DNA binding. Therefore the structural de-

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1 The abbreviations used are: bZIP, basic zipper; GST, glutathione S-transferase; DBD, DNA-binding domain; EBS, ETS-binding site; Py, polyomavirus enhancer; PBS, phosphate-buffered saline.
terminants of Erg are important for ternary complex assembly as well as required for transcriptional synergy. We thus propose that interdependent protein-protein and protein-DNA contacts regulate Erg-Jun/Fos-DNA complex assembly. The functional changes induced by these mutations define the location of a putative conserved Jun binding interface in the ETS domain.

EXPERIMENTAL PROCEDURES

Molecular Modeling—The proposed ETS-Jun/Fos-DNA complex model suggests that the recognition core of the DNA fusion protein DNA binding site 5'-TGACTCA-3'

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are bound to their DNA targets, we computed all possible nucleotide DNA structure adopting a standard double-stranded B-conformation. Then to investigate individual residues that could be involved in the protein-protein interaction when Elk-1 and Jun/Fos are simultaneously bound to their DNA targets, we computed all possible overlapping superimpositions of the selected Elk-1-DNA fragment (5'-GAAGTGT-3') along the 30-nucleotide standard DNA structure with the Jun/Fos heterodimer remaining fixed at its center. The set of structures used for DNA structure superimpositions were deoxyribose atoms C1', C2', C3', C4', and O4'. The root mean square values provided were around 1.5 Å. The ETS domain of the Elk-1-DNA complex was thus progressively advanced toward the Jun/Fos heterodimer. From all of the built ETS-Jun/Fos-DNA complexes, only one displays intermolecular contacts without steric clashes between protein backbones (see Fig. 2).

Cell Culture—ROS 17.2/8 (rat osteosarcoma) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Plasmid Constructions—The Erg deletion mutant expression vectors (constructs Erg (1-479) and Erg (307-479)) have been described previously (17). The amino acid substitutions and disrupted ETS domain derivatives were constructed by polymerase chain reaction using appropriate primers flanked by convenient restriction sites. Full details and primer sequences are available on request. Briefly, deletion mutants were obtained by polymerase chain reaction amplification and subcloned into pCRII (Invitrogen). Constructs were then cloned via EcoRI/BglII sites into pSG5 vector for further transcription and translation in vitro. To construct recombinant pGEX-2TK (Amersham Pharmacia Biotech), the DNA fragments encoding Jun, DBD, DBD-R367K, DBD-Y371V, and DBD-D374V were first generated by using polymerase chain reaction amplification of pSG5 hu-Jun and pSG5 hu-Ergps5, respectively, and subcloned into pCRII vector. The BamHI/EcoRI (Jun) or EcoRI (DBD) fragments were cloned in-frame into the cloning sites present within the pGEX-2TK polylinker. The amino acid junctions of the GST vector with the Jun or Erg sequences (in bold type) are: GST-Jun, SVGSMTAK; GST-DBD, GRPVYLGSGQQ; GST-DBD-R367K, IRPVYLGSGQQ; GST-DBD-Y371V, GRPVYLGSGQQ; and GST-DBD-D374V, GRPVYLGSGQQ. All constructs were verified by DNA sequencing.

Transfection and Luciferase Assays—The day before transfection, ROS 17.2/8 cells were plated at 50–60% confluence in 6-well plates. For transfection, cells were incubated with 1.0 µg of plasmid DNA and 4 µl of polyethyleneimine (Euromedex, Souffleweiseheim, France) for 6 h in 1 ml of OptiMEM and then in fresh complete medium. When necessary, pSG5 plasmid was used as a carrier. For reporter assays, detailed transfection conditions are indicated in the relevant figure legend. Cells were lysed 24 h after transfection and assayed for luciferase activity with a Berthold (Nashua, NH) chemiluminometer. Results presented are the means of at least five transfections.

In Vitro Protein Synthesis and Pull-down Assays—In vitro translated proteins were generated with rabbit reticulocyte in vitro transcription/translation system (TNT/T7/IIgma) and labeled with 50 µCi of [35S]methionine/50 µl of reticulocyte lysate. The translation products were visualized by SDS-polyacrylamide gel electrophoresis and quantified by PhosphorImager (Molecular Dynamics). The bacterial expression of GST constructs and the purification of GST fusion proteins were performed as described (17). Expression levels of the various GST fusion proteins were confirmed by Coomassie staining (data not shown) and by Western blot (see Fig. 5B).

For pull-down assays, 50 µl of glutathione-Sepharose beads (Amer sham Pharmacia Biotech) were incubated with 1 µg of GST, GST-Jun, GST-DBD, GST-DBD-R367K, GST-DBD-Y371V, or GST-DBD-D374V fusion proteins in NETN (20 mM Tris-HCl, pH 8, 500 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) for 1 h at 4 °C. Beads were washed three times with incubation buffer (12 mM HEPES, pH 7.9, 4 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM KCl, 1 mM EDTA) and resuspended in 30 µl of a mixture containing 32P-labeled protein expressed in reticulocyte lysate and incubation buffer. After 1 h at 4 °C, beads were washed six times with NETN and mixed with SDS sample buffer. The bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Electrophoretic Mobility Shift Assays and Western Blotting—The DNA binding reaction was performed at room temperature for 30 min in a total volume of 20 µl containing 20 mM Tris, pH 7.5, 80 mM NaCl, 2 mM dithiothreitol, 0.1% Triton X-100, 5% glycerol, and 5 µg/ml poly(dI-dC). As a probe, we used a double-stranded oligonucleotide corresponding to the polyomavirus enhancer (Py) 5'-GATCTTTAAGGGAGAATGACTACCTGACCGAGCTCGTCATC-3' or to an ETS consensus (EBS) 5'-GATCTTGAAACCCGAATGTTCTGAG-3' and labeled with [γ-32P]ATP at a concentration of 10,000 cpm/reaction. Protein-DNA complexes were resolved by 5–10% polyacrylamide gel containing 2% glycerol in TBE buffer. Autoradiography was performed on dry gels using an extra film to quench radioactivity arising from the 35S-labeled probe.

Before electrophoretic mobility shift assay, GST fusion proteins were visualized and quantified by Western blotting. Proteins were separated by SDS-polyacrylamide gel electrophoresis and then transferred to a Hybond-C Extra membrane (Amer sham Pharmacia Biotech) with a Bio-Rad dry blotter using 25 mM Tris-base, 192 mM glycine, and 20% (v/v) methanol as the transfer buffer. Membrane was blocked in PBS with 5% (w/v) dry milk for 1 h and stained with a rabbit antibody against the ETS domain of the human Erg protein (20) and a secondary goat anti rabbit/peroxidase antibody (Amer sham Pharmacia Biotech). Antibody incubations were performed for 1.5 h in PBS with 5% (w/v) dry milk followed by four 15-min washes in PBS with 0.1% Nonidet P-40. For detection we used the ECL chemiluminescent peroxidase substrate kit from Amersham Pharmacia Biotech. For transient transfection experiments, cell extracts were prepared from confluent 6-well plates. Cells were washed in cold PBS and harvested with Laemmli SDS sample buffer. Extracts were boiled for 5 min, and samples were resolved on 12% SDS-polyacrylamide gels and transferred to a Hybond-C Extra membrane (Amer sham Pharmacia Biotech) as for GST fusion detection.

RESULTS

Requirements for Erg-Jun/DNA Ternary Complex Assembly—In our previous study (17), we defined functional domains of the Erg transcription factor (Fig. 1A) and showed that Erg sequences 253–472, including the ETS domain, are necessary for Jun/Fos recruitment. To better characterize the Erg sequences important for Jun recruitment and Erg-Jun/Fos-DNA ternary complex assembly, we prepared a recombinant protein, named GST-DBD-Ergp55, comprising the ETS domain of Erg (amino acids 307–392 fused to GST) and Jun/Fos proteins (in vitro translated proteins translated in vitro using rabbit reticulocyte lysates) to perform GST pull-down assay. As shown in Fig. 1B, 35S-labeled Jun (lane 5) and the Jun/Fos heterodimer (lane 6), but not Fos alone (lane 4), bind specifically to immobilized intact GST-DBD but not to GST alone (lanes 7–9). Thus these results suggest that the ETS domain of Erg alone contains minimal sequences for recruiting the Jun/Fos heterodimer in vitro.

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We then performed electrophoretic mobility shift assays with the Py, known to contain adjacent EBS and AP-1 binding sites and to bind Ets and Jun/Fos proteins (21). Thus we incubated a 32P-labeled Py probe with purified GST recombinant proteins, corresponding to the Erg DNA-binding domain (GST-DBD), and with Jun/Fos proteins cotranslated in vitro. As shown in Fig. 1C, the Py probe formed, in the presence of both Erg-DBD and Jun/Fos proteins, a specific ternary complex that migrated more slowly than the secondary Jun/Fos-Py complex alone (compare lanes 2 and 4). A similar ternary complex was observed with full-length Erg (data not shown). Assembly of the ternary complex required both intact EBS and AP-1 binding sites because mutation of these sequences prevented ternary complex formation (data not shown). To make predictions about residues that could be involved in interactions between Erg and the Jun/Fos heterodimer, we modeled an ETS-Jun/Fos-DNA ternary complex (Fig. 2) using the available crystal coordinates of the Jun/Fos-DNA (18) and Elk-1-DNA complexes (19). The use of Elk-1-DNA instead of Erg-DNA complexes for which no crystallographic data are as yet available seemed appropriate; there is close similarity in the overall scaffold of the ETS domains because the winged helix-
The ETS-Jun/Fos-DNA Transcriptional Complex

FIG. 2. Model of the ternary complex ETS-Jun/Fos-DNA highlighting the putative protein interface between the two partners. This model was built on the basis of x-ray crystallographic coordinates of the Jun/Fos-DNA (18) and Elk-1-DNA complexes (19) using the molecular modeling program Insight II (Molecular Simulations Inc.). Residues located at the interface are depicted in sticks: Lys267 in Jun (K267), Arg62, Tyr66, and Asp69 in Elk-1 conserved in Erg respectively, Arg367, Tyr371, and Asp374. The backbone of macromolecules are represented by ribbons, Fos is in yellow, Jun is in red, the ETS domain of Elk-1 is in blue, and standard B-DNA is in pink. In addition, the LXXLL motif of the ETS domain is shown in green.

turn-helix motif and DNA-contacting residues are strongly conserved (19, 22). The rigid docking strategy followed to build the ETS-Jun/Fos-DNA ternary complex model is described under “Experimental Procedures.”

In our ETS-Jun/Fos-DNA model (Fig. 2), the two protein partners bind to the major groove on opposite sides of the DNA helix, positioning the helix $\alpha_3$ of the ETS domain and the N-terminal part of the Jun basic domain in close proximity. Principally we located Jun residue Lys267 and Elk-1 residues Arg62, Tyr66, and Asp69 conserved in Erg (Arg367, Tyr371, and Asp374) at the protein-protein interface. As observed in the different available crystal structures of ETS-DNA complexes (5, 19, 23, 24), the arginine residue Arg62 (Arg367 in Erg) is involved in hydrogen bonds with the DNA core sequence 5'-GGAA-3' and seems to be unavailable for supplementary interactions. Moreover the positive charge of its guanidinium group should not allow favorable interactions with the N-terminal basic domain of Jun, which is also positively charged. On the other hand, the phenol ring of Tyr66 (Tyr371 in Erg) involved in DNA contacts in the SAP-1-DNA complex structure (24) but not in Elk-1-DNA (19) and the carboxylate group of Asp69 (Asp374 in Erg), well accessible at the protein surface, could interact with the basic residue Lys267 of Jun (Fig. 2). However, if Tyr66 is highly conserved within the sequences of the ETS family and may thus be instrumental, Asp69 is frequently mutated (22) and thus may not play a major role in ETS-Jun/Fos complex formation (see below).

The Helix $\alpha_3$ of the ETS Domain Is Required for Interaction with Both DNA and Jun—Structural studies have revealed that the ETS domain forms a winged helix-turn-helix motif described to fold into a four-stranded anti-parallel $\beta$-sheet with three $\alpha$-helices (Fig. 3A), where helix $\alpha_3$ is the major DNA recognition component (19, 22, 24). Our model (Fig. 2) suggests that this helix $\alpha_3$ should also include sequences allowing the recruitment of the Jun/Fos heterodimer. To test this hypothesis, we prepared a set of differentially truncated polypeptides in the ETS domain and expressed in rabbit reticulocyte lysates. We then generated different Erg protein mutants in which either the first helix $\alpha_1$, both helices $\alpha_1$ and $\alpha_2$, or both $\beta_2$ and $\beta_4$ $\beta$-sheets of the ETS domain were deleted (Fig. 4A). As previously described (25), the integrity of the 85-amino acid ETS domain is necessary to bind an EBS, whereas Erg proteins lacking N-terminal helix $\alpha_1$, helices $\alpha_2$ and $\alpha_3$, or both $\beta_2$ and $\beta_4$ $\beta$-sheet failed to bind DNA (Fig. 4B). Strikingly our pull-down assays using glutathione S-transferase (GST) fusion proteins indicated that the ETS domain retained the ability to interact with GST-Jun (Fig. 4C, lanes 3, 6, 9, 12, 15, and 18), even when the $\alpha_1$ and $\alpha_2$ helices or the $\beta_2$ and $\beta_4$ $\beta$-sheets had been deleted and the DNA binding had been abolished (Fig. 4, B and C). Interestingly, these results revealed that deletion of the helix $\alpha_3$ did not affect Erg-Jun/Fos interaction, excluding the possibility that the conserved LXXLL motif, a potential protein-protein interface (26, 27), participates in the binding of Jun/ Fos. Moreover Erg and Jun proteins could form a complex independently of DNA binding because specific interactions between the Jun/Fos heterodimer and the ETS domain of Erg were also observed in the presence of ethidium bromide (data not shown), which is known to disrupt DNA-protein interactions (28). Thus although the ETS domain of Erg is the minimal Jun/Fos interaction domain, altering structural features such as the secondary structures can be without effect on the interaction with Jun. We have previously shown that deletion of the whole ETS domain abolishes Jun binding (17). Consequently all these results point out the DNA recognition helix $\alpha_3$ as the major ETS domain component for interaction with Jun protein.

Point Mutations in the Erg ETS Domain Impair Its Ability to Interact with Jun/Fos Heterodimer—We then attempted to locate in the helix $\alpha_3$ the amino acids more specifically involved in Erg-Jun/Fos interactions. Based on our molecular model of the ETS-Jun/Fos-DNA ternary complex, we identified essentially two Erg residues Tyr371 and Asp374 (Tyr371 and Asp374 in Elk-1) (Fig. 2). In the x-ray structure of the SAP-1-DNA complex (24), the tyrosine residue (Tyr371 in Erg) clearly participates in DNA recognition by contacting the thymine located on the complementary strand to the 5'-GGAA-3' core sequence. However this residue seems not strictly required for DNA binding because its substitution does not always abolish DNA recognition (22, 29). Note also that in the Elk-1-DNA complex crystal structure, no direct contacts of this residues are observed with DNA (19). Aspartic acid 374 of Erg (Asp69 in Elk-1) is highly conserved within the sequences of the ETS family and may thus be instrumental, Asp69 is frequently mutated (22) and thus may not play a major role in ETS-Jun/Fos complex formation (see below).

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As expected (22, 29), the mutation R367K abolished DNA binding (Fig. 5B, lane 3). On the other hand, the Y371V mutant exhibited a lower DNA binding in comparison with the wild type ETS domain, whereas D374V binds DNA with a stronger affinity (Fig. 5B, lanes 4 and 5, respectively). By in vitro com-
petition binding assays, we estimated that the Y371V mutant showed approximately a 5-fold decrease in DNA binding (data not shown). However, in the context of the full-length protein, this Y371V mutant entirely retained the transcriptional activity (Fig. 6B).

In GST pull-down assay, equivalent amounts of beads and bound Erg ETS domain fusion proteins based upon protein determination and staining of samples resolved by SDS-polyacrylamide gel electrophoresis were used, and expression levels of intact fusion proteins were monitored by Western blotting with an antibody against the ETS domain of the human Erg protein (Fig. 5B). Strikingly the R367K mutant, selected for noninteraction with DNA, scarcely interacted with Jun or the Jun/Fos heterodimer (Fig. 5C, compare lanes 5 and 6 with lanes 8 and 9). Conversely, although the mutation Y371V is not deleterious for DNA binding by Erg (Fig. 5B), it strongly decreases recruitment of the Jun moiety. On the other hand, the D374V substitution had no effect (Fig. 5B). As expected, the mutant R367K, which is defective for DNA binding, is also inactive for transcription (Fig. 6B). Despite the difference of their in vitro DNA binding capacities observed with respect to the wild type protein (Fig. 5B), the Y371V and D374V mutations did not affect Erg transcriptional activity of the Py enhancer. This apparent discrepancy cannot be a consequence of overexpression because all the mutant proteins were expressed in transfected cells at the same levels as the wild type Erg protein (Fig. 6D).

To exclude the possibility that these functional changes are due to an alteration of the overall folding of the mutants, we have taken advantage of the ability of Erg proteins to homodimerize (17). Indeed, we recently showed that the Erg proteins could form homodimers in vitro and that sequences required for this purpose are included in the ETS domain of the human Erg protein (17). Mutant proteins that fail to interact with Jun/Fos (Fig. 5, C and D) are still able to homodimerize (Fig. 5E). Taken together, these data indicate that amino acids Arg$^{367}$ and Tyr$^{371}$ of the ETS domain, but not Asp$^{374}$, play an important role in the recruitment of the Jun/Fos heterodimer by Erg, whereas the Erg homodimerization is independent of the mutated residues.

**Fig. 3.** The Erg ETS domain and its relationship to other ETS domain-DNA structures. A, amino acid sequences of the ETS domain of Erg and other members of the Ets family. The secondary structures indicated above are labeled with arrows. The strictly conserved residues that make DNA contacts are indicated with black boxes. The conserved Arg$^{367}$, Tyr$^{371}$, and Asp$^{374}$ residues are boxed, and the amino acid substitutions used in this study are indicated with arrows. B, amino acid sequences of the basic domain of Jun and Fos families (44). The variant amino acid Lys$^{267}$ of Jun singled out by our model and the corresponding arrows.
synergy fold value of about 2, as the Erg wild type. In contrast, R367K or Y371V substitutions abrogated Ets/AP-1 synergy, reflected by synergy fold values lower than 1. Thus physical interactions between the ETS domain of Erg and Jun are necessary for proper Erg-Jun/Fos synergistic activation of the Py enhancer.

**DISCUSSION**

In this report we have explored the role of specific residues located at the protein interface of the Erg-Jun/Fos-DNA complex, focusing on their functional importance in regulating gene expression. Previously we and others showed that the ETS domain was critical for interaction with Jun/Fos (14–17). By molecular modeling and mutational studies, we demonstrated here that the helix $\alpha_3$ of the ETS domain, known to be involved in DNA recognition, is also involved in Jun/Fos recruitment. Two substitutions in this helix (Y371V and D374V in Erg) preserve DNA binding in vitro, whereas a third (R367K) abolishes both DNA binding and Jun/Fos recruitment. However when co-expressed with the Jun/Fos heterodimer, the Y371V mutant did not display a transactivation cooperation similar to that observed with the D374V mutant or the wild type. Thus Tyr$^{371}$ of Erg is clearly required for the interaction with Jun/Fos and also for the synergistic activation of the Erg-Jun/Fos-DNA complex.

Overall Conserved Structure of the Winged Helix-turn-Helix-bZIP-DNA Complex?—Our initial goal was to distinguish between ETS domain functions involved in DNA binding and protein-protein interaction. We firstly reasoned that the structural determinants required for Erg-Jun/Fos complex formation are located away from the DNA recognition helix $\alpha_3$. Therefore we investigated the role of the conserved LXXLL motif located in helix $\alpha_1$ (Figs. 2 and 3A) as a potential protein-protein interface because this motif has been described to be sufficient for ligand-dependent interaction between nuclear receptors and coactivators (26, 27). We thus introduced the L320A mutation in this motif of the Erg protein. However this substitution together with the deletion of the helix $\alpha_1$ (Fig. 4C) has no effect on interaction with Jun or on DNA binding (data not shown). Surprisingly our experimental data coupled with our molecular model rather suggest that helix $\alpha_3$ defines the location of a Jun binding interface in the ETS domain. Interestingly the recent crystal structure of the winged helix-turn-helix IRF-2 protein (32) lead to the proposal of a model of bZIP-IRF-DNA complex formation at the interferon-$\beta$ enhancer (32, 33); both cases are reminiscent of ours (Fig. 2). It is noteworthy that the regions of closest approach between ATF-2/Jun and IRF-3 are the bZIP N terminus and the IRF DNA recogni-

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**Fig. 4. Identification of Erg ETS domain regions essential for interaction with Jun/Fos.** A, structure of the Erg deletion mutants. Amino acid numbers of encoded proteins are indicated for each construct. Interactions observed in B and C are summarized on the right. The minimal domain (helix $\alpha_1$) that is involved in interaction with Jun is indicated. B, band shift analysis of wild type and Erg protein mutants. 5–10 $\mu$g of in vitro translated proteins were incubated with the labeled ETS consensus oligonucleotide 5'-GATCTTGAAACCGAAGTTCCGAG-3'. The empty vector pSG5 utilized as a control is indicated. F, free probe. C, GST pull-down assays showing the interaction between GST-Jun and Erg, using the deletion constructs shown in A.
tion helix (32, 33). Differences in amino acid sequence between ATF-2, Jun, and Fos and between IRF-1, IRF-3, and Ets may underlie the specific structural and transcriptional properties of the distinct complexes.

In good agreement with this idea, we note that the Jun family members have been shown to interact with Ets proteins, whereas the Fos family members do not share this property (14). This suggests that amino acids shared by Jun family members but not present in Fos family members could interact with the Ets proteins. A mapping of the minimal Jun domain involved in Ets-Jun interactions located the interaction within residues 263–282 in the basic domain of Jun. The Lys267 Jun residue singled out by our model is precisely located within this domain and thus represents a suitable candidate for the bridging with Erg through Tyr371 (Fig. 2). Intriguingly alignment of this restricted domain from different Jun and Fos proteins revealed that residue Lys267 is conserved in Jun proteins but not in Fos proteins where it is replaced by Arg143 (Fig. 3B). We thus attempted to create a Fos compensatory mutant R143K that could restore interaction with the ETS domain. However the mutation R143K alone is not sufficient (data not shown), suggesting that residues at the periphery of Lys267 of Jun are also instrumental (Fig. 3B).

Identification of Mutations That Disrupt the Erg-Jun/Fos Interaction without Abolishing DNA Binding by Erg—A key amino acid for Jun/Fos recruitment by Erg is an almost invariant tyrosine residue present in the DNA recognition helix α₃ (Figs. 2 and 3A). In our ternary complex model, this residue is located close to the basic domain of Jun, suggesting that it could play a direct role in interactions between these two proteins. The substitution Y371V in the ETS domain alters the affinity of Erg for an EBS probe (Fig. 5B), suggesting that the aromatic residue could interact directly with DNA, as observed in the SAP-1-DNA complex with the corresponding residue Tyr65 (24). However this mutation drastically hinders the Erg-Jun/Fos complex formation (Fig. 5D). Hence because of a reori-
The tyrosine residue could prefer to interact with the Jun protein in the Erg-Jun/Fos-DNA ternary complex. Recently, a certain flexibility was indeed reported for this residue because its side chain clearly adopted two different conformations in Elk-1-DNA (19) and SAP-1-DNA complexes (24). In this context, Tyr371 of Erg could act as a discriminator of protein interactions/coactivation and DNA binding.

According to the available crystal structures of ETS domains (5, 19, 23, 24), the absolutely conserved residue arginine 367 of Erg is clearly crucial for DNA binding. It is thus not surprising that mutation R367K abolishes DNA binding in vitro and consequently showed no transcriptional activation. Initially we envisioned an indirect role for this residue in Erg-Jun/Fos complex assembly. Indeed, in SAP-1-DNA complex structure (24), the guanidinium group of Arg61 (Arg367 in Erg) is engaged in hydrogen bonds with the DNA core sequence 5′-GGAA-3′ but also makes close contacts with the phenol ring of Tyr65 (Tyr371 in Erg). The mutation of the conserved arginine residue by a lysine could induce a rearrangement of the contiguous aromatic side chain and then modify interactions of Erg protein with Jun/Fos heterodimer.

The ETS DNA-binding Domain: One Domain, Multiple Functions—Partnerships between transcription factors are required for specific binding and control of gene expression. Although these interactions are quite common, the structural basis for complex formation and identification of instrumental amino acids have only been determined for a small number of examples (5, 8, 30, 31, 34–36). For instance, the isolated DNA-binding domain of GATA proteins mediates physical interactions with FOG (34, 36). However, all the mutant GATA-1 proteins that impaired FOG interaction retained the ability to bind DNA (35, 36). In our case, unlike GATA-1, mutants of Erg, such as R367K, selected for noninteraction with DNA, also prevent association with the Jun/Fos heterodimer. Similarly the ability of Ets proteins to be recruited by Pax-5 coincides with the presence of a specific aspartic acid (Asp374 of Erg in

**FIG. 6. Functional cooperation between Jun/Fos and various Erg mutants.** A, structure of the constructs used in transient transfections. The indicated point mutations in the ETS domain of Erg were introduced in the context of the full-length protein and cloned into pSG5. B, ROS cells were cotransfected with the polyomavirus enhancer-Luc reporter and different Erg mutants as indicated in the absence (B) or in presence (C) of Jun/Fos. Results are expressed as fold activation relative to basal promoter activity. D, analysis of the Erg mutants expression by Western blotting. ROS cells were transfected with 1 μg of different Erg mutants as indicated. Cells were harvested as described under “Experimental Procedures,” and extracts were analyzed by SDS-gel electrophoresis and probed with anti-Erg antibody (1:1000) and developed by ECL (Amerham Pharmacia Biotech) according to the manufacturer’s protocol.
this study) within the ETS DNA-binding domain (30, 31). However, Ets protein (SAP-1, which has a valine residue instead of an aspartic acid), which impaired Pax-5 binding, retained the ability to bind DNA (30, 31). Importantly mutation of this position had no effect on Erg-Jun/Fos complex formation (Fig. 5D), suggesting that the structural determinants required for this complex overlap with but can be uncoupled from those required for Pax-5/Ets complex formation.

The idea of considering the ETS DNA-binding domains as protein-protein interaction domains has begun to emerge recently. In particular, a growing number of protein-protein interactions have been shown to be mediated by the Pu-1 ETS domain. Indeed, Pu-1 has previously been found to interact with Jun family members (14, 37), proteins of the C/EBP family (38, 39), and erythroid zinc finger transcription factor GATA (40–42). Strikingly the binding surfaces for Erg and Pu-1 on Jun/Fos appear to differ significantly (41). In our study (Fig. 4C), unlike Pu-1, the β/β′ domain does not seem to be involved in the recruitment of Jun. A functional significance of this apparent discrepancy could be overcome by the unconserved position Tyr771 in Erg versus Asn236 in Pu-1 (Fig. 3A). As we strongly demonstrated the instrumental role of this Tyr771 residue in Erg for Jun recruitment as well as transcriptional synergy (Figs. 5D and 6C), we concluded that the adaptation of different mechanisms for protein-protein interactions reflects the structural and functional diversity of Ets proteins. Interestingly, recent work also suggests that the binding surfaces for Fli-1 and Elk-1 on serum response factor are different (43). Different mechanisms for protein-protein interactions reflects the structural determinants required for Pax-5/Ets complex formation.

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