Ten ERK-related Proteins in Three Distinct Classes Associate with AP-1 Proteins and/or AP-1 DNA

N. Vinay Kumar‡§ and Lori R. Bernstein†¶

*This work was supported by Grant R29 CA73783 from the National Institutes of Health and Grant 96G-821 from the Texas Affiliate of the American Heart Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

From the ‡Department of Pathology and Laboratory Medicine, Texas A & M University System Health Science Center, College Station, Texas 77843-1114

Received for publication, April 25, 2001, and in revised form, June 22, 2001
Published, JBC Papers in Press, June 28, 2001, DOI 10.1074/jbc.M103677200

Ten ERK-related proteins ("ERPs"), including ERK2, that are stably associated in vivo with AP-1 dimers composed of diverse Jun and Fos family proteins. These complexes have kinase activity. We designate them as "class I ERPs." We originally hypothesized that these ERPs associate with DNA along with AP-1 proteins. We devised a DNA affinity chromatography-based analytical assay for DNA binding, the "nucleotide affinity preincubation specificity test recognition" (NAPSTER) assay. In this assay, class I ERPs do not associate with AP-1 DNA. However, several new "class II" ERPs do associate with DNA. p41 and p44 are ERK1/2-related ERPs that lack kinase activity and associate along with AP-1 proteins with AP-1 DNA. Class I ERPs and their associated kinase activity thus appear to bind AP-1 dimers when they are not bound to DNA and then disengage and are replaced by class II ERPs to form higher order complexes when AP-1 dimers bind DNA. p97 is a class III ERP, related to ERK3, that associates with AP-1 DNA without AP-1 proteins. With the exception of ERK2, none of the 10 ERPs appear to be known mitogen-activated protein kinase superfamily members.

Regulatory transcription factors are of central importance in mediating cellular responses to environmental stimuli by coordinately regulating genes encoding proteins and enzymes that implement the responses. Activator protein-1 (AP-1)1 is a transcription factor that binds to and regulates genes containing TGAg/TCA consensus cis-regulatory elements (referred to here as "AP-1 DNA-binding sites" or "AP-1 DNA") (1, 2) that generally lie within gene promoter regions. Regulation by AP-1 has been demonstrated in diverse cellular processes, including growth, differentiation, tissue remodeling, and apoptosis (Ref. 3 and references therein).

Altered expression and DNA binding of AP-1 subunits, and inappropriate transactivation of AP-1-dependent effector genes, are events implicated in the pathogenesis of numerous cancers and in cardiovascular, neurological, and other disease states (4–6). AP-1 activation is a pivotal event in mediating cancer susceptibility and neoplastic transformation in response to endogenous and extracellular stimuli, including hormones, growth factors, tumor promoters, and many other carcinogenic agents (Refs. 7–9 and see Ref. 3 for review). Many genes associated with neoplastic transformation harbor AP-1 sites, including genes encoding matrix-degrading enzymes, differentiation factors, and mitogenic agents.

AP-1 is a dimer composed of proto-oncogene products encoded by the jun and fos families. The Jun family consists of c-jun, junB, and junD, and the Fos family consists of c-fos, fra-1, fra-2, and fosB. Dimers can be Jun family-Jun family homodimers or Jun family-Fos family heterodimers, generating 18 possible complexes via leucine zipper dimerization motifs within the Jun and Fos subunits. AP-1 dimers bind DNA via a basic region immediately adjoining the leucine zipper, forming a composite region in the proteins referred to as the "bZIP" motif (see Ref. 10 for review). Gene expression is regulated by transactivation domains within the AP-1 subunits, which modulate the efficiency of RNA polymerase II transcriptional initiation.

The transactivation and DNA binding activities of AP-1 are modulated by protein kinase cascades that terminate in phosphorylation of Jun and Fos family proteins. Members of the mitogen-activated protein kinase superfamily ("MAP kinases") are thought to be responsible for phosphorylation of AP-1 proteins in vivo (see Ref. 11 for review). Ligand-activated receptors indirectly stimulate kinase activity of MAP kinase kinases (MAPKKs) that activate MAP kinase kinases (MAPKKs), which in turn activate MAPKs to phosphorylate their targets, often transcription factors such as Jun and Fos (see Refs. 12 and 13 for review). The MAP kinase superfamily is composed of several subfamilies. Each subfamily consists of a discrete signaling module with distinct MAPKKK and MAPKK components. The most well characterized members of the ERK subfamily are ERK-1 and ERK-2 and, to a lesser degree, p63/ERK-3 and human p97/ERK3 (14–16). The Jun kinase (JNK)/stress-activated protein kinase (SAPK) family includes three major members, JNK1, JNK2, and JNK3. The p38 family includes p38α, p38β, p38γ, and p38δ (Ref. 13 and references therein). Other MAPKs include ERK4, ERK-5/BMK1, ERK6, and ERK7 (17–21). ERKs are primarily activated by mitogenic agonists such as TPA, epidermal growth factor, or fibroblast growth factor, whereas JNK/SAPKs are primarily activated by stress factors such as UV light, tumor necrosis factor, osmotic...
and heat shock, and inflammatory agents (12). p38 MAPKs are activated by endotoxic lipopolysaccharide and, like JNK/SAPKs, by environmental stress, osmotic shock, and inflammatory agents (13). A system of several parallel MAPK signal transduction pathways has thus emerged.

MAPKs regulate a number of transcription factors, including Elk/TCF, NFAT, ATF, and AP-1. ERKs and JNK/SAPKs mediate activity of AP-1 in response to mitogenic and stress factors, respectively. ERKs phosphorylate c-Jun, JunD, Fra-1, FosB, and JNKs phosphorylate c-Jun and JunD (22–26). JNK binding to docking sites on c-Jun and JunD has been reported (25). Both ERKs and JNKs regulate AP-1 transactivation and mediate AP-1-dependent neoplastic transformation (27–30).

Interactions between AP-1 and many other proteins have been identified. Multiple members of the bZIP AP-1 superfamily, and of the nuclear hormone receptor transcription factor superfamily, have been found in association with AP-1. Other transcription factors that interact with AP-1 include NFAT, Ets, YY1, TATA-binding protein, and Myo D (10, 31). Coactivators and repressors that bind to AP-1 include CBP/p300, JAB1 along with eight other proteins in the COP 9 complex, ASC-2, and JDP2 (32–34). In this paper we refer to this sequence as the AP-1-DNA-binding site or AP-1 DNA. This sequence and the inverse complementary oligo were custom-synthesized by Macromolecular Resources (Fort Collins, CO). Binding of annealed, double-stranded biotinylated oligo to streptavidin beads (Roche Molecular Biochemicals, Indianapolis, IN) was performed according to the manufacturer’s instructions.

AP-1 DNA Affinity Chromatography—AP-1 DNA affinity chromatography was performed as described by Lee et al. (1) with major modifications designed to achieve rapid analytical scale isolation of labile and multicomponent protein-DNA complexes. For small scale experiments, as little as 30 µg of input NE protein and 6 µg of DNA on beads were used (1:5 ratio of DNA:input protein). For large scale experiments, 3–5 mg of NE protein and 150–250 µg of DNA on beads were used (1:20 ratio of DNA:input protein). Whole nuclear extracts (NE) were dialyzed into Buffer Z (0.1 M KCl, 25 mM HEPES, pH 7.8, 12.5 mM MgCl₂, 1 mM dithiothreitol, 20% glycerol, v/v, 0.1% w/v Nonidet P-40, 0.1 µM ZnCl₂, 5 mM NaF, 1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride). AP-1 and associated proteins were then isolated by single-step batchwise AP-1 DNA affinity chromatography to promote maximal stability and detection of associated proteins. Dialyzed NE was incubated for 75 min at 4 °C on a rotating clip wheel in the presence of 6 µg/ml poly(dI-dC) with AP-1 affinity beads. Beads were centrifuged for 1 min in an Eppendorf centrifuge at 4 °C, and the supernatant was removed. For small scale assays, beads were washed three times with Buffer Z, bound material was eluted by boiling the beads in SDS sample buffer, and the boiled material was directly loaded in SDS-PAGE. For large scale assays, beads were washed five times in Buffer Z and then AP-1 and associated proteins eluted in Buffer Z containing 1 M KCl by twirling on a rotating clip wheel for 30 min at 4 °C.

The NAPSTER Assay—To assess specificity of binding to the AP-1 DNA sequence, we developed an assay that we termed the nucleotide affinity greenuincubation specificity test recognition assay (the NAPSTER assay). The NAPSTER assay consists of a matched set of three samples. For sample I, whole NE is directly chromatographed batchwise with AP-1 DNA beads. For sample II, NE is preincubated for 15 min at 4 °C with a 2.5-fold molar excess of wild type AP-1 DNA oligo (relative to moles of DNA on the beads) before batchwise DNA affinity chromatography. For sample III, NE is preincubated for 15 min at 4 °C with a 2.5-fold molar excess of mutant oligo (5'-agcaggaanatagagctacagc-3'; mutant AP-1 core sequence GAAGTCT, mutations underlined), before chromatography. After chromatography, beads are washed and directly loaded in SDS-PAGE or subjected to elution procedures as described above.

SDS-PAGE and Immunoblotting—SDS-PAGE and immunoblotting were performed as described (39, 43).

Peptide Competition Assays—Peptide competition assays for immunoblotting and immunodepletions were performed using a 25-fold molar excess of peptide as described (39, 43).

Preparation of Recombiant Proteins—A full-length recombinant human p97/ERK3 cDNA sequence (rERK3) was cloned into the pET3C vector (Stratagene, La Jolla, CA). Thirty cycles of polymerase chain reaction were performed to amplify a linear BambH属于EcRo-rERK3 DNA fragment containing the full-length ERK3 gene from plasmid.
pGEX2T-ERK3, using forward primer 5'-agggttcattcgaggaatat
-g-3' and reverse primer 5'-ttgtggtcagctagcggatt-3', which enabled in-frame insertion of the gene into the NdeI site of the vector. Recombinant hERK3 was expressed in BL21 Codon Plus bacteria (Stratagene, Inc., La Jolla, CA) according to the manufacturer's instructions.

Glutathione S-transferase-JunD (GST-JunD; see Ref. 45), a mouse JunD fusion construct, was expressed in DH5α bacteria. Expressed JunD was purified from bacterial extracts by GST affinity chromatography with glutathione-agarose beads (Sigma) and eluted with 15 mM glutathione (Sigma). pgCTD, a GST fusion construct containing the mouse gene encoding the C-terminal domain of RNA polymerase II, ("CTD"); see Ref. 46) was expressed, and GST-CTD fusion protein was purified on the GST affinity column.

V8 Proteolytic Digestion Assay of p97 and Recombinant ERK3—V8 protease digest analyses were performed on p97 protein isolated by batchwise DNA affinity chromatography from 20 mg of nuclear extract protein. Proteins isolated on affinity beads were eluted in 1 M KCl and precipitated in 10% w/v trichloroacetic acid at 4 °C. Precipitated protein was run in denaturing and reducing SDS-PAGE, and the p97 band was excised from the gel. Recombinant human ERK3 was also run in SDS-PAGE and similarly excised for V8 analyses. Excised p97 and recombinant human ERK3 bands were subjected to V8 protease digestion with 0.04 units of endoprotease Glu-C (V8 protease; Roche Molecular Biochemicals) per lane, according to procedures described previously (39, 47).

Immunodepletion Assay—Samples either underwent two rounds ("double depletion") or three rounds ("triple depletion") of immunodepletion. For double depletions, ~3.2 mg of NE per sample was dialyzed against Buffer Z and incubated for 90 min with an antibody mix consisting of 20 μg of α-c-Jun, 20 μg of αJunD (Santa Cruz Biotechnology), and 10 μg of αJunB (KG), all of which had been dialedyzed against Buffer Z for 45 min at 4 °C. Samples were then incubated with 25 μl of protein A-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4 °C with constant mixing. The procedure was repeated a second time to obtain double depleted samples. For triple depletions, ~5.1 mg of dialyzed NE was preincubated for 90 min at 4 °C with an antibody mix consisting of 20 μg of α-c-Jun, 20 μg of αJunB, 20 μg of αJunD, 10 μg of αJunB (KG), 20 μg of αFra-1, and 20 μg of αFra-2, followed by incubation with protein A-Sepharose for 1 h at 4 °C. This procedure was repeated twice to obtain triple depleted samples. Control samples did not contain antibody mix but underwent depletion with protein A-Sepharose alone. Samples were then preadsorbed to reduce nonspecific binding by batchwise DNA affinity chromatography with beads containing streptavidin-linked mutant anti-Jun or anti-oligo.

Immunodepleted samples were subjected to the NAPSTER assay.

Immunoprecipitations—For two-dimensional gels and immunodepletion assays, immunoprecipitations were performed as described (see above and Ref. 39). For kinase assays and Western blots, Dynal protein A magnetic beads (Dynal, Oslo, Norway) were preadsorbed for 30 min at room temperature with 5 μg of antibody in 0.1 ml of Phosphate Buffer, pH 8.0. Beads were incubated for 1 h with 1.25–500 μg of histone H3 (Roche Molecular Biochemicals), or 10 μg of histone H1 (Roche Molecular Biochemicals), or 1 μg of recombinant c-Jun (Upstate Biotechnologies, Inc.), or 1 μg of recombinant c-Fos, 10 μg of GST-CTD, or GST-JunD; 10 μl of [γ-32P]ATP mixture (1 μl of 10 mCi, 6000 Ci/mmol; Amersham Pharmacia Biotech) diluted with proteins eluted in 9 μl of 75 μM MgCl2 and 500 μM ATP in Assay Buffer), and 30 μl of Protein A-Sepharose for 15 min at 30 °C.

In Vivo Metabolic Labeling—In vivo metabolic labeling of JB6 C30 7b cells with Translabel (ICN, Inc., Costa Mesa, CA) was performed as described (39). In vivo metabolic labeling of HT29 cells with Tran32P-label was also performed as described (39), except that labeling was done overnight in cells plated at 6 × 106 cells/150-mm tissue culture dish.

Two-dimensional Gels—Two-dimensional non-equilibrium pH gradient gel electrophoresis (NIEPAGE two-dimensional gels) was performed as described (39). pH 3.5 to 10 and pH 5.0 to 7.0 ampholines were from Pierce.

RESULTS

Immunoprecipitations with Anti-Jun and -Fos Antibodies Detect ERK2 and Numerous Associated ERK-related Proteins Bound in Vivo to Numerous AP-1 Dimerization Partners—To determine whether ERK-related proteins (ERPs) interact in vivo with AP-1 components, we performed immunoprecipitations of 32S-metabolically labeled mouse epidermal JB6 C30 7b and human HT29 colon adenocarcinoma whole cell extracts with MAP kinase and AP-1 antibodies, followed by comparative two-dimensional gel electrophoresis (two-dimensional gels). As shown in Fig. 1, the PAN-ERK antibody αERK1-III immunoprecipitates ERK2, since a major spot immunoprecipitated by αERK1-III has identical mobility to ERK2 immunoprecipitated by specific anti-ERK2 (compare protein spots in Fig. 1, panels 1 and 2; see Ref. 39). Immunoprecipitation of whole cell extracts with αERK1-III followed by Western blots with specific antibodies against ERK2 corroborates these data (39). Several other ERPs immunoprecipitated by ERK1-III are also detected and are designated by their molecular weights as p49, p65, and p100 as well as p38, p36, and p33 (B-D, respectively in Fig. 1, where this p38 protein is not the p38 MAP kinase, see below).

These ERPs are specifically immunoprecipitated with αERK1-III since antibody preincubation with the ERK1-III peptide antigen, but not a heterologous peptide, abolishes immunoprecipitation of these proteins (panel 1, Fig. 1). Spots X, Y, and E are ERK-related proteins specifically immunoprecipitated with αERK1-III that are not coimmunoprecipitated with antibodies against AP1 species.

Immunoprecipitation with αc-Fos and with αFra-1 immunoprecipitates c-Fos and Fra-1 and coprecipitates AP-1 partners c-Jun and JunD (panels 1 and 2 of Fig. 1). Ac-Jun immunoprecipitates AP-1 dimers containing the dimerization partner Fra-2 in JB6 cells and c-Fos in HT29 cells (panel 2, Fig. 1). Comparison of protein spots coprecipitated along with c-Jun, c-Fos, and Fra-1 to proteins immunoprecipitated by the PAN-ERK MAP kinase antibody αERK1-III shows that many ERK-related proteins are coimmunoprecipitated with Fra-1 and c-Jun. We collectively refer to these seven ERPs as class I ERPs. These include proteins p100 (with c-Fos), p65 (with Fra-1), p49 (with c-Fos and c-Jun), proteins ERK2, p38 (B), p36 (C), and p33 (D) (with c-Jun, c-Fos, and Fra-1), and p65 (with c-Jun, c-D) (with c-Jun and Fra-1). Coimmunoprecipitation of ERPs p49, ERK2, and p38 with c-Jun and c-Fos is also observed with extracts from human HT29 colon adenocarcinoma cells, demonstrating that ERAP-1 complexes also exist in this cell type (Fig. 1, panel 2). p49 and p38 were also detected in one-dimensional SDS-PAGE by Western immunoblotting of αJunD immunoprecipitates from HT29 cells (not shown), thus corroborating the results from two-dimensional gels. These data demonstrate the association of multiple ERPs with diverse AP-1 components in both JB6 epidermal cells and HT29 colon adenocarcinoma cell types.

Several ERPs are in the general molecular weight range of known MAPKs. We therefore performed experiments to determine whether these ERPs share identity with these MAPKs, particularly those for which there is sufficient homology within subdomain III (the region of ERK1 against which the peptide
III fail to display reactivity when immunoblotted with specific p38 MAPKs; however, ERPs immunoprecipitated with and p33 are plausibly within the molecular weight range of the molecular weight range of ERK1, p49 and ERK1 are readily dis- unlikely candidate ERPs. Although p49 is in the general molecular weight range of ERK7, a 61-kDa protein (21). The possibility that p65 is ERK7 cannot be ruled out, although the homology of ERK7 to the ERK1-III peptide sequence is weak. Taken together from these data it appears that, with the exception of ERK2, most if not all of the ERPs bound to AP-1 are previously unidentified proteins rather than known MAP kinases.

ERK2 forms stable complexes \textit{in vivo} with AP-1. However, the observation that multiple additional proteins immunologically related to MAP kinases bind AP-1 proteins \textit{in vivo} is both unique and perplexing. To explain the multiplicity and stability of these complexes and understand their functions, we postulated that AP-1 and ERPs form regulatory higher order complexes at the transcriptional regulatory site of AP-1 DNA binding. The first prediction of this hypothesis is that ERPs will be detectable in association with the AP-1 DNA along with AP-1 proteins.

Our initial efforts to detect ERPs in AP-1-DNA complexes were to perform electrophoretic mobility supershift assays with radiolabeled AP-1 DNA oligonucleotides and anti-ERK antibodies. No supershift was observed (not shown). Since the absence of a supershift is not evidence that an antigen is absent from a complex, we sought to develop an alternative means of assaying for protein-DNA interactions on a micro scale. The method of preparative DNA affinity chromatography (1, 49) was adapted to an analytical scale to detect ERPs in association with the AP-1 DNA-binding site. A single step batchwise affinity chromatographic method was devised for whole nuclear extracts (NE) using affinity beads attached to AP-1 DNA. This is followed by SDS-PAGE and immunoblotting with anti-AP-1 and anti-MAPK antibodies.

Fig. 1. ERK2 and several other ERK-related proteins interact \textit{in vivo} with multiple AP-1 transcription factor subunits. Immunoprecipitations were performed with whole cell extracts from \textit{in vivo} metabolically $^{35}$S-labeled JB6 and HT29 cells with the indicated antibodies. Panel 1, A–C, JB6 cells. A, αPAN-ERK (αERK1-III); B, αPAN-ERK + preincubated ERK1-III competitor peptide; C, αcFos-(75–155). Panel 2, A–D, JB6 cells; E, HT29 cells. A, ERK1/2 and ERK5/BMK1 (PAN anti-ERK αMAP kinase); C, αFRA-1-(1–276); D, αJun-(948–4); E, αJun-(948–4). For this and all subsequent figures, $M_r$ is for molecular weight standards.

antibody was made) to expect immunological reactivity with the αERK1-III antibody. ERK3, ERK4, and the JNK family members JNK 1–3 have low homology to the ERK1-III peptide and would not be expected to be reactive with αERK1-III antibody. Immunoblotting of αERK1-III immunoprecipitates with anti-JNK failed to detect JNK (not shown). JNKS are thus unlikely candidate ERPs. Although p49 is in the general molecular weight range of ERK1, p49 and ERK1 are readily distinguishable in SDS-PAGE (not shown). ERPs p49, p38, p36, and p33 are plausibly within the molecular weight range of the p38 MAPKs; however, ERPs immunoprecipitated with αERK1-III fail to display reactivity when immunoblotted with specific op38 MAPK antibodies (not shown). Several ERPs are also in the molecular weight range of the ERK6 MAPK (38 kDa; alias p38γ). Although we were able to detect ERK6 in cell extracts of human A673 rhabdomyosarcoma cells, ERK6 was undetectable in NE of HT29 cells. Although the p100 ERP is in the general molecular weight range of ERK5/BMK1 (110 kDa; see Ref. 48), the homology between the ERK1-III peptide and ERK5/BMK1 is relatively weak. The p65 ERP is in the general molecular weight range of ERK7, a 61-kDa protein (21). The possibility that p65 is ERK7 cannot be ruled out, although the homology of ERK7 to the ERK1-III peptide sequence is weak. Taken together from these data it appears that, with the exception of ERK2, most if not all of the ERPs bound to AP-1 are previously unidentified proteins rather than known MAP kinases.

The NAPSTER Assay, an Analytical Scale Assay for Specific-DNA Interactions—To ascertain the binding specificity of AP-1 and associated proteins, we devised a simple assay that we term the NAPSTER assay. The assay enables identification of proteins that interact specifically with the DNA-binding site on the affinity beads and distinguish these from proteins that bind nonspecifically. In the NAPSTER assay, nuclear extracts are either directly subjected to DNA affinity chromatography or are preincubated with wild type (wt) or mutant (mut) AP-1 oligo prior to batchwise DNA affinity chromatography. For analyses of AP-1 composition, samples from NAPSTER isolation are subsequently subjected to Laemmli SDS-PAGE and Western immunoblotting with anti-AP-1 antibodies. One would predict that proteins that bind specifically to the AP-1 DNA-binding site will undergo specific competition by wt but not mut oligo, whereas proteins that bind nonspecifically will not be competed differentially by the two sequences. By these means specific binding of c-Jun and JunD to the AP-1 DNA-binding site was detected, thus demonstrating the efficacy of the NAPSTER assay method (Fig. 2A). Specific binding to AP-1 DNA-binding sites from two different genes, the Gibbon ape leukemia virus long terminal repeat (GALV-LTR) and human...
Ten ERK-related Proteins Bind AP-1 Proteins and/or AP-1 DNA

Fig. 2. AP-1 dimers from HT29 adenocarcinoma cell nuclei interact specifically with affinity beads containing the AP-1 DNA and contain multiple Jun and Fos partners. A, AP-1 dimers bind specifically to AP-1 affinity beads in the NAPSTER assay. HT29 cells were treated for 90 min with TPA, and NE were prepared from harvested cells. DNA affinity chromatography with 30 μg of protein from whole NE was either performed directly (–) or after preincubation with a 2.5-fold molar excess of wild type (wt) or mutant (mut) AP-1 oligonucleotides derived from the GALV-LTR. NE (5 μg) directly loaded in SDS-PAGE. NE, –, wt, and mut nomenclature are also used for NAPSTER assays in all subsequent figures. Proteins bound on the DNA affinity beads were run in SDS-PAGE by direct loading of beads boiled in SDS sample buffer. Proteins were transferred by Western blot and immunoblotted with specific antibodies against each of the seven AP-1 subunits, JunA, JunB, and JunD (1 μg/ml each). c-Jun and JunD are shown with arrows. B and C, titration competition in the NAPSTER assay demonstrates specific binding of AP-1 transcription factor to AP-1 DNA beads. B, lane C, control incubation of NE + beads without preincubated free oligos. Numbers above the triangles indicate increasing concentrations of free wild type (wt) or mutant (mut) oligo, expressed as fold molar excess of free oligo relative to bound wt oligo on beads. Bracket indicates signal of JunD proteins in Western/ECL immunodetection. C, quantitation of B. Similar results for quantitative preincubation were obtained in three independent experiments; graphed results are from a representative experiment. D, AP-1 bound to beads contains c-Jun, JunD, c-Fos, Fra-1, and Fra-2 but not JunB or FosB. DNA affinity chromatography was performed, and affinity beads containing bound AP-1 were directly loaded in SDS-PAGE and subjected to Western transfer and immunoblotting with specific antibodies against each of the seven AP-1 transcription in a multiscreen apparatus (Bio-Rad).

Collagenase I, was demonstrated (Fig. 2 and data not shown). The typical yield of Jun proteins bound to the beads relative to that present in unfractionated NE was between 30 and 50%. Association of AP-1 with DNA on the beads was blocked as a function preincubation with increasing concentrations of wt AP-1 oligo but not mut oligo (Fig. 2, B and C).

To determine which AP-1 family members participate in these complexes, we visualized the material bound to the AP-1 DNA beads by Laemmli SDS-PAGE/Western with antibodies against all seven AP-1 subunits. Binding to AP-1 DNA was detected for JunD, c-Jun, c-Fos, Fra-1, and Fra-2 but not for JunB or FosB (Fig. 2D). Western analyses of whole NE detected expression of the AP-1 components that bind the DNA but did not detect expression of JunB and FosB, even when large quantities were loaded (not shown). Specificity of Western detection was verified since preincubation of antibodies with their cognate antigenic peptides inhibited their detection, whereas preincubation with unrelated peptide sequences did not (not shown).

Fig. 3. ERK-related proteins p41 and p44 associate along with AP-1 at the AP-1 DNA-binding site. A, NAPSTER assay with αERK1-III. NE, nuclear extract directly loaded in SDS-PAGE. NE directly loaded in SDS-PAGE. Samples were eluted by boiling beads in SDS sample buffer, loaded in SDS-PAGE, and subjected to Western transfer and immunoblotting with the PAN-ERK antibody αERK1-III using 2 μg/ml αERK1-III antibody. B, αERK1-III antibody specifically detects p44 and p41 ERPs. 1 μg of NE per NAPSTER assay sample was subjected to AP-1 DNA affinity chromatography, run in SDS-PAGE, transferred by Western blot, and immunoblotted with αERK1-III antibody that had been preincubated with a 25-fold molar excess of antigenic peptide (+pep), “nonspecific” peptide (ns against amino acids 303–325 of rat ERK3), or no peptide (–). C, ERPs p41 and p44 are not the MAP kinases ERK1 and ERK2. 375 μg of NE per sample were subjected to the NAPSTER assay. Proteins were visualized by immunoblotting with αERK1 antibody (which recognizes ERK1 and ERK2). ERK1 and ERK2 proteins are indicated with arrows.
with mut AP-1 oligo, but not if they were preincubated with wt AP-1 oligo. Antibody preincubation with antigenic ERK3 D23 peptide (amino acids 303–325 of rat ERK3) abolished detection of p97, whereas preincubation with a heterologous peptide (amino acids 353–567 of rat ERK3) did not. Therefore, p97 is an ERP that interacts specifically with the AP-1 DNA sequence. We refer to p97 as a class III ERP.

ERPs p44 and p41 Are Not ERK1 or ERK2—p44 and p41 have apparent molecular weights close to the MAP kinases ERK1 and ERK2, and αERK1-III is capable of recognizing ERK1 and ERK2 (50) (Fig. 3A, lane NE). We therefore sought to determine whether p44 and p41 are ERK1 and ERK2. To do this we performed the NAPSTER assay and immunoblotted with a second anti-ERK1 antibody that specifically recognizes ERK1 and ERK2 but is not a PAN-ERK antibody (sc-93, Santa Cruz Biotechnology). Whereas ERK1 and ERK2 were readily detected in nuclear extracts, no signal was detected in material input NE was tested (not shown). p38 was detectable, even when 400 μg of protein from NE per sample. Controls for specific detection of p97 protein by anti-ERK3 D23 antibody were performed by antibody preincubation with 25-fold molar excess of competitor peptide. −Pep, immunoblotting performed without preincubation of ERK3 D23 antibody with peptide. +Pep, preincubation with D23 peptide. NS, preincubation with 115 peptide (amino acids 353–367 of rat ERK3).

Fig. 4. The ERK3-related protein p97 associates specifically with AP-1 DNA affinity beads. The NAPSTER assay was performed using 300 μg of protein from NE per sample. Controls for specific detection of p97 protein by anti-ERK3 D23 antibody were performed by antibody preincubation with 25-fold molar excess of competitor peptide. −Pep, immunoblotting performed without preincubation of ERK3 D23 antibody with peptide. +Pep, preincubation with D23 peptide. NS, preincubation with 115 peptide (amino acids 353–367 of rat ERK3).

The 97-kDa ERK3-related Protein Is Not Human p97/ERK3—The 97-kDa protein human p97/ERK3 contains an epitope recognized by the ERK3 D23 antibody. Therefore, when we detected a protein of 97 kDa associated with AP-1 and DNA, our initial hypothesis was that it was the human 97-kDa protein p97/ERK3 (hERK3). To test this we performed the NAPSTER assay with several additional antibodies against diverse epitopes within hERK3. None of the additional antibodies detected a specific signal in the vicinity of 97 kDa. To determine whether this was due to a lack of sensitivity of these antibodies or to the absence of the predicted ERK3 epitopes in p97, we expressed bacterial recombinant hERK3 protein (15), and we compared the Western detectability of recombinant hERK3 to that of affinity-purified cellular p97 from the NAPSTER assay.

Full-length recombinant hERK3 protein (rhERK3) from bacterial extracts was readily detectable by Coomassie staining (Fig. 5A) and by Western immunoblotting with several antibodies against distinct epitopes in hERK3 (Fig. 5, B–D). rhERK3 ran as a protein with a molecular mass of 105 kDa, compared with an apparent molecular mass in SDS-PAGE of 97 kDa reported by Zhu et al. (15). p97 isolated from NE and detected by αERK3 D23 migrated faster than the recombinant p97/hERK3 (Fig. 5B). However, whereas the other two ERK antibodies also detected rhERK3, they failed to detect a specific band isolated in the NAPSTER assay in the molecular mass range of p97, even when 400 μg of NE was used per sample (Fig. 5, C and D). A protein that ran in the vicinity of ERK3 but somewhat more slowly at 110 kDa (“p110”) was detected in nuclear extracts with anti-ERK3-C and anti-ERK3-F (Fig. 5, C and D). This may be a posttranslationally modified (perhaps phosphorylated) form of hERK3. Detection of rhERK3, p97, and p110 was specifically competed with specific but not heterologous peptides when immunoblotted with all three αERK3 antibodies (not shown). Taken together these data do not support the hypothesis that the p97 protein that associates with the AP-1 DNA-binding site is the human p97/hERK3 protein.

To determine more definitively whether p97 and ERK3 are the same or different proteins, we isolated rhERK3 and p97 and performed comparative V8 protease mapping. p97 was isolated by DNA affinity chromatography of 20 mg of protein from whole nuclear extract. Isolated p97 and p97/hERK3 from bacterial extracts were gel-purified by excision of isolated bands in SDS-PAGE and digested with endoprotease Glu-C (“V8 protease”). As shown in Fig. 5E, the V8 digestion patterns of p97 and rhERK3 are strikingly different. Distinct V8 digestion patterns for p97 and rhERK3 were observed even when a variety of V8 protease concentrations was tested (not shown). These data demonstrate that p97 is not human p97/ERK3.

Immunoprecipitates Containing AP-1 and ERPs Contain Kinase Activity, but No Kinase Activity Is Detected in Association with AP-1 DNA—To determine whether immune complexes containing AP-1 and ERPs may contain stably associated kinase activity, we performed in vitro kinase reactions using the classic MAP kinase substrate MBP. As shown in Fig. 6, immune complexes isolated by immunoprecipitation from NE with oc-Jun, oc-Fos, and ocJunD contained MBP kinase activity. As expected, immunoprecipitates isolated with an antibody against ERKs 1 and 2 (PAN-ERK antibody αERK1-III; Fig. 6), and against ERK3 (αERK3 D23, Fig. 6, and αERK3 I15; not shown) also displayed kinase activity. Kinase activity for samples immunoprecipitated with AP-1 and ERK antibodies was significantly higher than control samples incubated with normal rabbit serum (for antibodies against full-length proteins) or for peptide antibodies when preincubated with excess peptide antigen competitor before immunoprecipitation (not shown). We conclude that kinase activity is stably associated with intracellular AP-1 transcription factors.

To explain the presence of multiple ERPs in complexes with...
Ten ERK-related Proteins Bind AP-1 Proteins and/or AP-1 DNA

Fig. 5. The AP-1 DNA-associated protein p97 is not human ERK-3. A, expression of recombinant p97/ERK3 in bacteria. Extracts were made from E. coli transformed with the bacterial expression construct pET3C-ERK3 encoding full-length ERK3. Samples were run in SDS-PAGE and stained with Coomassie Blue. —, no isopropyl-1-thio-β-D-galactopyranoside induction; +, with isopropyl-1-thio-β-D-galactopyranoside induction. B, specific detection of recombinant human ERK3 and p97 with anti-human ERK3 D23 antibody. C and D, endogenous human ERK3 is not detectable specifically associated with AP-1 DNA. C, immunoblotting with anti-ERK3 C against amino acids 427–442 of rat ERK3; D, immunoblotting with anti-ERK3 F, a specific antibody against 543–721 of human p97/ERK3, an epitope in the C terminus of human p97/ERK3 that is not present in rat ERK3. * in B–D denotes a band from nuclear extracts running slightly above recombinant ERK3 that is detected with anti-rat and anti-human ERK3 antibodies and may be native human ERK3. E, p97 is not p97/ERK3. p97 was isolated by AP-1 DNA affinity chromatography of 20 mg of whole nuclear extract. p97 and recombinant human p97/ERK3 were pre-purified in SDS-PAGE. Comparative V8 protease digestion of p97 and rhERK3 isolated as gel slices from SDS-PAGE were performed. Gel slices for V8 protease digestion were overlaid with 0.04 units of endoproteinase Glu-C (V8 protease), run through the gel, and subjected to Western transfer and immunoblotting with ERK3 D23 antibody. S, sample from DNA affinity chromatography; R, rhERK3. Arrows indicate locations of digestion products.

AP-1, we hypothesized that they participate in AP-1-DNA complexes and regulate transcriptional activation by AP-1, perhaps by phosphorylating Jun and Fos family members and/or other DNA-binding proteins proximal to the AP-1-DNA-binding site. An initial prediction of this hypothesis is that kinase activity will be detected in association with the AP-1 DNA. To test this hypothesis, kinase activity assays of NAPSTER-isolated protein complexes associated with AP-1 DNA was performed. Bound proteins were eluted with wt AP-1- oligo to eliminate nonspecific kinase activity bound to the beads. Protein kinase assays were performed on the eluted proteins with various substrates. The substrates tested were MBP (see Fig. 6), c-Jun, JunD, c-Fos, histone H1, histone H3, and the C-terminal domain of RNA polymerase II (CTD), which is phosphorylated by MAP kinases in vitro and whose phosphorylation modulates RNA polymerase II transcriptional activity (51–53). No detectable kinase activity for any substrate tested was associated with the AP-1 DNA-binding site, although specific association of AP-1 subunits and ERPs with AP-1 DNA was detected in the same experiments by Western blots, and positive control kinase activity with recombinant enzymes was observed (ERK1 enzyme for MBP, CTD, and c-Fos substrates; PKC enzyme for histone H1 and H3 substrates; JNK1 enzyme for MBP, c-Jun and JunD substrates; Fig. 6 for MBP and data not shown). No specific MBP kinase activity was detected in reanimated kinase assays (54) with MBP impregnated in SDS-PAGE gels, using NAPSTER isolated material from 400 μg of HT29 whole NE (not shown). These data show that although kinase activity is associated with AP-1 proteins when they are not bound to DNA, it is lost upon DNA binding.

Association of c-Jun, p41, and p44 with AP-1 DNA Is TPA-inducible but Association of JunD and p97 Is Not—We compared the intracellular levels and association of AP-1, p41, p44, and p97 isolated from NE in TPA-treated versus untreated cells (Fig. 7A). Levels of c-Jun in the nucleus were TPA-inducible, whereas levels of JunD were not. AP-1 DNA binding of JunD and c-Jun correlated with levels of expression, with induction of DNA binding observed for c-Jun but not JunD. Like c-Jun, ERPs p44 and p41 showed significant increases in association with DNA after TPA treatment. Since expression of p41 and p44 was undetectable in nuclear extracts, it is not evident whether the induced participation of p41 and p44 in the complexes is due to induced expression of p41 and p44 proteins, increased affinity for the AP-1 complex, or increased recruitment by c-Jun protein within the complex, although we speculate that the latter may be the case. In contrast, the association of p97 with DNA was not TPA-inducible, since the amount of p97 associated with the AP-1 DNA-binding site was equal in TPA-treated and untreated cells.

Identification of Multicomponent Class II ERP-AP-1 Protein-AP-1 DNA Complexes—We hypothesized that class II ERPs are docked to the DNA by the AP-1 dimer. However, there also exists the possibility that association of ERPs with DNA is independent of AP-1 proteins. To determine if DNA association by ERPs is dependent or independent on AP-1 proteins, we immunodepleted nuclear extracts of AP-1 proteins prior to AP-1 DNA affinity chromatography. Since Fos binds the AP-1 DNA only when heterodimerized with Jun, depletion of Jun subunits should abolish the binding of all AP-1 complexes, and should also deplete proteins whose association with the AP-1 DNA are dependent on AP-1 proteins. A single round of immunodepletion with α-Jun plus α-JunD depleted 80–90% of AP-1. Two rounds of depletion with these antibodies efficiently depleted Jun subunits to levels that were virtually undetectable (Fig. 7B). Two rounds of AP-1 immunodepletion also eliminated association of p41 and p44 ERPs as evidenced...
in Western immunoblotting with αERK1-III of NAPSTER-isolated material (Fig. 7B). These data demonstrate that association of p41 and p44 ERPs with the AP-1 DNA-binding site is dependent upon the presence of AP-1 transcription factor. Based on these data it is likely that p41, p44, and AP-1 form multicomponent complexes at the AP-1 DNA-binding site.

Association of ERp p97 with DNA Is Independent of AP-1 Transcription Factors—We also performed immunodepletion to test the hypothesis that p97 participates in a multicomponent complex with AP-1 protein and AP-1 DNA. NAPSTER assays were performed with double depleted NE and immunoblotted with αERK3 D23 antibody. There was no decrease in the amount of p97 specifically associated with AP-1 DNA in extracts that had undergone two rounds of immunodepletion of AP-1, relative to undepleted samples (Fig. 7B). This suggested that association of p97 is independent of AP-1 proteins. After two rounds of depletion Jun subunits were undetectable, whereas some Fos family subunits were still detectable (most likely due to greater sensitivity of the Fos antibody). To rule out the possibility that p97 was associating indirectly with AP-1 DNA via residual undepleted AP-1, or in a manner uniquely Fos-dependent but Jun-independent, we performed three rounds of depletion with antibodies against both Fos family and Jun family proteins. AP-1 DNA affinity chromatography showed that c-Fos, Fra-1, Fra-2, and JunD were depleted to undetectable levels in the triple depleted samples (Fig. 7C). Association of p97 with DNA was unaffected by complete depletion of Jun and Fos subunits. These data demonstrate that specific association of p97 with AP-1 DNA-binding site is independent of AP-1 protein binding. The class III ERP p97 is thus distinguishable from the other ERP classes by virtue of its relatedness to ERK3 (rather than ERK2) and its association with DNA in the absence of AP-1 proteins.

DISCUSSION

In this report we describe 10 ERK-related proteins that consist of three distinct classes of proteins. These classes are defined by differences in their interactions with AP-1 proteins and the AP-1 DNA-binding site. As summarized in Table I, class I ERPs are ERK1/2-related proteins that bind in vivo with AP-1 proteins but do not associate with the AP-1 DNA. Class II ERPs, also ERK1/2-related, bind to AP-1 proteins and associate with AP-1 DNA in a manner that is dependent upon AP-1 proteins. The class III ERP p97 is related to ERK3 and associates with the AP-1 DNA without evidence of association with AP-1 proteins. Both class II and class III ERPs may interact with DNA either indirectly via protein-protein contacts or directly with the DNA. We have also designated a number of MAP kinase superfamily members as class IV MAPKs based upon the observation that they are not detected in stable association with AP-1 proteins or AP-1 DNA in the NAPSTER assay system. These include the MAPKs ERK1, ERK3, ERK4, ERK5/BMK, and ERK6. In our studies, JNK1 and JNK2 also qualify as class IV ERPs, although other laboratories have detected JNK-Jun associations in communoprecipitation assays when epitope-tagged genes encoding AP-1 and JNK components were transiently overexpressed in cultured cells (22–23, 25, 55, 56). Since JNKs are kinases for Jun, such interactions are to be expected and may be transient in nature, since communoprecipitation of endogenously expressed JNKs and Juns have not been observed by our group or reported by others.

We have detected a diverse variety of AP-1 transcription factor subunits in association in vivo with an equally diverse variety of class I ERPs. The detection of multiple Jun and Fos subunits in these complexes is a new observation. AP-1 subunits found in association with ERPs include c-Jun, JunD, c-Fos, Fra-1, and Fra-2. Only JunB and FosB were not detected in these complexes. The variety of ERPs in the complexes includes the following seven proteins: p100, p65, p49, p38, p36, p33, and the MAP kinase ERK2. ERPs were detected in association with AP-1 in both mouse epidermal JB6 cells and human HT29 colon carcinoma cells. Since AP-1 complexes are dimers of Jun-Fos or Jun-Jun family members, these data indicate that many different types of AP-1-ERP complexes are present in cells and that the existence of these complexes occurs in cell types originating from different tissues, both cancerous and noncancerous.

What are the functions of these complexes? Why do so many different types of ERPs and AP-1 dimers interact with one another, and why are the complexes so stable? If the interac-
tion between ERP and AP-1 is only that of an enzyme and substrate, the observed multiplicity of stable protein-protein interaction would not be expected. To address this paradox we formulated a hypothesis that we have called the ERP-AP-1 docking hypothesis. By this hypothesis, AP-1 and ERPs form functional higher order complexes at the site of AP-1 DNA binding and regulate gene transcription by modulating communication with other transcription factors or accessory factors proximal to the AP-1 DNA-binding site via protein phosphorylation and/or protein-protein interactions. The first prediction of the hypothesis is that ERPs will be detectable in association with AP-1 proteins bound at the AP-1 DNA-binding site. Studies in other laboratories have shown that when bound to DNA, AP-1 can interact with neighboring DNA-binding proteins within the regulatory promoter region, including Smad proteins (57), Ets proteins (58), and NFAT (59, 60). However, to date no proteins related to ERK MAP kinases have been found in association with AP-1 at the DNA-binding site.

To test this prediction we performed the NAPSTER assay and identified three proteins that associate with the AP-1 DNA: the class II p41 and p44 proteins, immunologically related to ERK1 and ERK2 MAPKs, and the class III p97 protein, immunologically related to ERK3 MAPK. Whereas p97 associated with DNA independently of AP-1 proteins, association of p41 and p44 required AP-1 proteins, suggesting the presence of p41 and p44 in higher order complexes with AP-1 and DNA. The class II p41 and p44 ERPs thus fulfill the first prediction of the ERP-AP-1 docking hypothesis. NAPSTER screening demonstrated no detectable binding of known MAPK proteins at the AP-1 DNA-binding site, including MAPKs ERK1, ERK2, ERK3, ERK4, ERK5/BMK1, ERK6, Jnk1, and Jnk2.

### TABLE 1

| Class | Proteins | Binds AP-1 proteins | Association with AP-1 DNA | Related to | Associated kinase activity |
|-------|----------|---------------------|--------------------------|------------|---------------------------|
| I, 7 proteins | p33, p36, p38, ERK2, p49, p65, p100 | Yes | N.O.* | N.O. | ERK1/2 | Yes |
| II, 2 proteins | p41, p44 | Yes | Yes | N.O. | ERK1/2 | N.O. |
| III, 1 protein | p97 | N.O. | N.O. | Yes | Human ERK3 | N.O. |
| IV | ERK1, ERK3, ERK4, ERK5/BMK1, ERK6, Jnk1, Jnk2 | N.O. | N.O. | N.O. | ERKs and Jnks | Yes |

* N.O., not observed.
Ten ERK-related Proteins Bind AP-1 Proteins and/or AP-1 DNA

ERK3, ERK5/BMK, ERK6, p38, JNK1, and JNK2. As described above, with the exception of ERK2, none of these proteins were detected in association with AP-1 dimers in the absence of DNA either.

ERPs p100, p65, p49, p38 (which is not MAPK p38; see “Results”), p36, p33, and ERK2 are detected in association with AP-1 proteins by coimmunoprecipitation under solution conditions that are unfavorable to protein-DNA interactions, and these proteins were not detected in association with AP-1 DNA in the NAPSTER assay. Based upon their molecular weights (with the exception of ERK2, which also has been ruled out; see Fig. 3C), these ERPs and p41 and p44 are different proteins. We detected no evidence of p41 and p44 bound to AP-1 proteins in the absence of DNA.

The second prediction of the ERP-AP-1 docking hypothesis is that we should detect kinase activity in association with AP-1 proteins bound to DNA. This prediction was not borne out. We detected kinase activity associated with AP-1 proteins only when AP-1 proteins were not bound to DNA. While the possibility cannot be ruled out that kinase activity is associated with AP-1 DNA that was not detected due to the wrong choice of substrate or insufficient quantities of enzyme, this appears improbable since many substrates were tested, and NAPSTER kinase assays were performed with large quantities of starting material.

It is likely that ERK2 is a major source of AP-1 protein-associated kinase activity from AP-1 immunoprecipitates since ERK2 is the only one of the ERPs associated with AP-1 that had measurable kinase activity in renatured kinase assays with impregnated myelin basic protein substrate. Kinase activity either falls off of AP-1 dimers or is turned off upon association with DNA. We consider the former possibility more likely since ERK2 is detected in association with AP-1 proteins only when they are not bound to the DNA. From these data our new working hypothesis is that the class I ERPs including ERK2 kinase activity are bound to AP-1 in the absence of DNA, and they disengage from AP-1 and are replaced by the class II ERPs p41 and p44, thus forming a new higher order complex when AP-1 proteins bind the DNA. We also hypothesize that class II p41 and p44 ERPs associate with DNA in a manner that is dependent upon c-Jun rather than JunD, since p41, p44, and c-Jun but not JunD exhibit TPA-dependent induction of DNA binding. We speculate that the class I ERPs that bind AP-1 in the absence of DNA function to repress its DNA binding and that the class II ERPs that associate along with AP-1 proteins with the DNA modulate its transactivating function. Future experiments will be done to test these notions. It is unclear how many of these respective components compose these protein-protein and protein-DNA complexes or whether the contacts of class II ERPs with DNA are direct or indirect. Further studies involving molecular cloning of the genes encoding these numerous class I and class II proteins, and other studies to identify DNA-binding proteins proximal to the AP-1 DNA-binding site with which functional interactions with ERPs may be occurring, are ongoing to address these interesting questions.

In initial experiments on this project we attempted to detect evidence of ERP-AP-1 DNA interactions with gel supershift assays employing the αERK1-III antibody, but none were detectable under a variety of assay conditions. In hopes of circumventing this problem, we devised the small scale specificity-controlled NAPSTER assay, adapted from large scale DNA affinity chromatography. The assay succeeded in identifying two proteins cross-reactive with αERK1-III that were missed by the gel supershift method. The NAPSTER assay is performed at much higher concentrations of protein and DNA than the gel shift assay, and so may more effectively promote the stability of specific low affinity interactions and multicomponent protein-DNA complexes. Because the NAPSTER assay is rapid, specific, and capable of detecting interactions that are missed by gel supershift assays, we believe that it will be useful to investigators as an alternative method for studying protein-DNA interactions on an analytical scale. We also expect that the assay will be generally applicable to transcription factors other than AP-1 and to other DNA-binding proteins.

As indicated above, several laboratories (22–23, 25, 55–56, 59) have detected physical associations between Jun kinases and Jun proteins, in vitro assays, by immunoprecipitation of epitope-tagged transiently cotransfected c-Jun and JNK proteins in cultured cells and by yeast and mammalian two-hybrid analyses. c-Jun amino acid sequences involved in these interactions include amino acids 31–47, a region that is deleted in v-Jun (55), and the C-terminal region between amino acids 182 and 256 (56). Gel mobility shift assays performed by May et al. (56) employing an AP-1 DNA oligonucleotide and high concentrations of purified recombinant c-Jun and JNK proteins suggested the possibility of ternary complexes of AP-1, JNK, and DNA. However, appropriate mutant AP-1 oligonucleotide controls for DNA binding were not performed in these experiments, so a specific requirement for the AP-1 consensus DNA sequence for JNK binding was not demonstrated. In our hands using the NAPSTER assay, although JNK bound DNA containing an AP-1 sequence, binding was nonspecific since preincubation with wild type oligos failed to block JNK binding (not shown).

Some comparisons may be made between the MAP kinase BMK5/ERK1 and class I or class II ERPs. Recently, several laboratories (61–63) have found that ERK5/BMK1 is a regulator of MEF2 transcription factors. ERK5/BMK1 positively regulates gene transactivation by MEF2, phosphorylates MEF2 in vitro, and physically interacts with MEF2. Although binding interactions with MEF2-ERK5/BMK1 complexes to DNA have not been demonstrated, a transactivation domain in ERK5/BMK1 has been identified that modulates MEF2 gene transactivation, and the kinase domain within ERK5/BMK1 is required for positive ERK5/BMK regulation of MEF2 activity. If ERK5/BMK were to associate along with MEF2 with DNA, it would fall into a different functional class than class I or class II ERPs since in the latter cases kinase activity is observed only in ERPs that are not associated with DNA.

Recent work by Janulis et al. (64) has uncovered a new MAP kinase-related protein which is, like our class III ERP, named p97, based upon its molecular weight. The p97 protein identified by Janulis et al. (64) is detected by anti-phospho-ERK and anti-PAN-ERP antibodies, has kinase activity, modulates downstream Raf signaling, and binds to Raf protein. It is unlikely that the p97 molecule discovered by Janulis et al. (64) is the same molecule as our p97 because our p97 protein has no kinase activity, and PAN-ERP antibodies do not cross-react with our p97 protein.

The class III p97 protein stands out as the only ERP that is immunologically related to ERK3, and the only one that associates with the DNA without requiring the involvement of AP-1 proteins. To date, proteins in the general molecular weight range of p97 have not been identified that associate with AP-1 proteins. Because p97 does not require AP-1 dimers to associate with the DNA, we performed the NAPSTER assay to determine whether other AP-1 superfamily members that can bind the minimal AP-1 consensus sequence could have specifically bound the AP-1 site along with a putative coassociated p97.

---

2 L. Bernstein, unpublished data.
Specific binding of CREB, ATF, Maf, and other cap 'n collar AP-1 superfamily members to the AP-1 DNA was not detected. 3 p97 therefore appears to be a novel species that either associates with the AP-1 DNA in the absence of other factors or binds with other factors in a new multicomponent complex that has not been described previously. It is possible that p97 is a new transactivating or transrepressing protein that competes with AP-1 for binding the AP-1 site. Future work, including molecular cloning of p97, is ongoing to characterize the functions of this interesting protein and any proteins that may be associated with it when it binds to the AP-1 DNA.

Acknowledgments—We gratefully acknowledge the gifts of reagents kindly provided by other investigators: αJun(κG) and GST-JunD from Dr. M. Cobb (University of Texas Southwestern Medical Center, Dallas); αERK3 and hERK3 expression construct from Dr. J Flier (Charles A. Dana Research Institute, Glasgow, Scotland, UK); αH9251 from Dr. K. Gardner (National Institutes of Health, Bethesda); AP-1 superfamily members to the AP-1 DNA was not detected.3 We thank Dr. Douglas Ferris for technical assistance and Drs. Kenneth Ramos, Julian Liebowitz, David Peterson, and Michael Klade for helpful discussions and comments on the manuscript.

REFERENCES

1. Lee, W., Mitchell, P., and Tjian, R. (1987) Cell 49, 741–752.
2. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Kornbluth, S., Coburn, N. H., and Colburn, N. H. (1999) J. Biol. Chem. 274, 35297–35300.
3. Aronheim, A., Zandi, E., Hennemann, H., Elledge, S. J., and Karin, M. (1997) Mol. Cell. Biol. 17, 3009–3012.
4. Karin, M. (1996) Nature 383, 453–457.
5. Albanaese, C., D’Amico, M., Reutens, A. J., Ferrari, A., and Wasylyk, B. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8599–8603.
6. Powers, C., Krutzsch, H., and Gardner, K. (1996) J. Biol. Chem. 271, 1102–1106.
7. Abe, J., Kusuohara, M., Ulevitch, R. J., Berk, B. C., and Lee, J. D. (1996) J. Biol. Chem. 271, 16586–16590.
8. Kadonaga, J. T., and Tjian, R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5889–5893.
9. Sanghera, J. S., Peter, M., Nigg, E. A., and Pelech, S. L. (1992) Mol. Cell. Biol. 12, 775–787.
10. Kato, Y., Kravchenko, V. V., Tapping, R. I., Han, J., Ulevitch, R. J., and Lee, R. J. (1998) Mol. Cell. Biol. 18, 3099–3103.
11. Bensaude, O. (1994) Biochem. Biophys. Acta 1266, 1051–1057.
12. Bonnet, P., Vigneron, M., Bensaude, O., and Dubois, M. F. (1999) Nucleic Acids Res. 27, 4399–4404.
13. Kameshita, I., and Fujisawa, H. (1989) Annu. Rev. Biochem. 58, 139–143.
14. Dai, T., Rubie, E., Frankin, C. C., Kraft, A., Bensaude, O., and Dubois, M. F. (1999) J. Biol. Chem. 274, 33439–33445.
15. Zhang, Y., Peng, X. H., and Derynck, R. (1998) Nature 394, 909–913.
16. Gutman, A., and Wasylyk, B. (1990) EMBO J. 9, 2246–2250.
17. Kishimoto, S., Fujisawa, H., and Sato, T. (1991) J. Biol. Chem. 266, 1325–1329.
18. Frame, M. C., Wilkie, N. M., Darling, A. J., Chudleigh, A., Pintzas, A., Lang, W., and Desai, P. (1997) Mol. Cell. Biol. 17, 4205–4213.
19. Bernstein, L. R., and Walker, S. E. (1999) Biochem. Biophys. Acta 1488, 263–280.
20. Davies, R., and Kubes, P. (1999) Mol. Cell. Biol. 19, 2235–2241.
21. Kober, B., Wang, J., and Derynck, R. (1997) J. Biol. Chem. 272, 17054–17059.
22. Yang, C. H., Bamba, K., and Kishimoto, S. (1998) J. Biol. Chem. 273, 30527–30532.
Ten ERK-related Proteins in Three Distinct Classes Associate with AP-1 Proteins and/or AP-1 DNA
N. Vinay Kumar and Lori R. Bernstein

J. Biol. Chem. 2001, 276:32362-32372.
doi: 10.1074/jbc.M103677200 originally published online June 28, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103677200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 64 references, 29 of which can be accessed free at http://www.jbc.org/content/276/34/32362.full.html#ref-list-1