THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 34, Issue of August 24, pp. 32362–32372, 2001
Printed in U.S.A.

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

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

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We have identified seven 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/cTCA 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 kinase kinases (MAPKKKs) 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

* 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.

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[1] The abbreviations used are: AP-1, activator protein-1; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; ERP, ERK-related protein; NAPSTER assay, nucleotide affinity preincubation specificity test recognition assay; NE, nuclear extract; oligo, oligonucleotide; wt, wild type; mut, mutant; Fra, Fos-related antigen; PAGE, polyacrylamide gel electrophoresis; α, antibody; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; FBS, fetal bovine serum; GALV, gibbon ape leukemia virus; GST, glutathione *S*-transferase; JNK, Jun N-terminal kinase; SAPK, stress-activated protein kinase; LTR, long terminal repeat; MBP, myelin basic protein; MOPS, 4-morpholinepropanesulfonic acid; CTD, C-terminal domain.

and heat shock, and inflammatory agents (13). 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, Fra-2, and 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 (32–37). The retino-blastoma protein pRb also binds AP-1 proteins (38). The diverse array of regulatory molecules that interact with Jun-Fos complexes underscores the exquisite circuitry dedicated to controlling AP-1 in its pivotal position as a regulator of essential cellular functions.

Previously we identified several ERK-related proteins (ERPs), including the ERK2 MAP kinase, that bind *in vivo* to AP-1 complexes containing c-Jun and c-Fos (39). In the present study we have found that five Jun and Fos family members form complexes with seven distinct ERK2-related ERPs, and these ERP·AP-1 complexes have associated kinase activity. To account for the multiplicity and stability of these complexes, we hypothesized that in addition to a simple enzyme-substrate relation, ERP·AP-1 complexes could interact with genes containing transcription regulatory AP-1 DNA recognition sequences. If this is the case, we should be able detect multicomponent ERP·AP-1·DNA complexes. To test this hypothesis, we devised a simple DNA affinity chromatography-based analytical assay, the "NAPSTER" assay, for specific association with DNA sequences. By these means we identified p41 and p44, two additional ERPs related to ERK2 that associate with the AP-1 DNA along with AP-1 proteins. In contrast, the first seven ERPs bind AP-1 proteins only when they are not bound to the DNA. We also identified p97, a 10th ERP related to ERK3, that associates with AP-1 DNA without AP-1 proteins. We therefore describe three distinct classes of ERPs as follows: seven class I ERPs that bind AP-1 dimers without DNA, two class II ERPs that associate along with AP-1 with DNA, and one class III ERP that associates with AP-1 DNA without AP-1 dimers.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies and corresponding peptide antigen competitor peptides used for immunoblotting and immunodepletions included αc-Jun, αJunB, αJunD, αc-Fos, αFra-1, αFra-2, αFosB, αp38α, αJNK, αERK1, αERK5/BMK1, αERK6, and αERK3 D23 (which also recognizes human p97/ERK3) against amino acids 303–325 in rat ERK3, all of which were from Santa Cruz Biotechnology. Unless otherwise noted, antibodies used in experiments were from Santa Cruz Biotechnology. Other antibodies included anti-rat ERK3 "C" against amino acids 427–442 of rat ERK3, anti-human ERK3 "F" against amino acids 543–721 of human ERK3, and anti-rat ERK3 "I15" against amino acids 353–367 of rat ERK3. Antibodies from other sources include αJunD ("KG") against full-length mouse JunD (40), αc-Jun-(948-4) against the C-terminal 82 amino acids of avian c-Jun (41), mouse monoclonal anti-ERK1/2 antibody 1B3B9 (Upstate Biotechnologies, Inc., Lake Placid, NY), αFos-(75–155) against amino acid residues 75–155 of avian v-Fos (Upstate Biotechnologies, Inc.), and αFra-1-(1–276) against amino acids 1–276 of Fra-1 (42), all of which were used in immunoprecipitations and two-dimensional gels. Anti-ERK1-III is against amino acids 63–98 in subdomain III of rat ERK1 (Upstate Biotechnologies, Inc.).

Reagents—Reagents and supplies not described herein were purchased from vendors cited in Bernstein and Walker (43).

Cell Culture—Human HT29 adenocarcinoma cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). For routine passage, HT29 cells were plated at 1:20 in T25 flasks in Dulbecco's modified Eagle's medium (Mediatech Cellgro, Herndon, VA) containing 10% heat-inactivated fetal bovine serum (FBS, Summit Biotechnology, Inc., Fort Collins, CO) and passaged every week with media changes after 4 days. JB6 Cl307b cells were grown and passaged as described (44).

Preparation of Nuclear Extracts—HT29 cells for nuclear extracts ("NE") were plated in 150-mm tissue culture dishes (Nunc, Inc., Naperville, IL) at a density of 6×10^6 cells per dish in Dulbecco's modified Eagle's medium with 10% FBS and allowed to grow for 5 days with one media change 4 days after plating. Cells were routinely treated for 90 min with 10 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma) in fresh media containing 10% FBS, unless otherwise noted. Cells were then harvested, and NE was prepared as described (43).

Preparation of AP-1 DNA Affinity Beads—Biotinylated AP-1 oligonucleotide ("oligo") derived from nucleotides from the gibbon ape leukemia virus-long terminal repeat (GALV-LTR), contained a wild type AP-1 sequence (5′-agccagagaaatag**tgagtca**acagc-3′). 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 $MgCl_2$, 1 mM dithiothreitol, 20% glycerol, v/v, 0.1% v/v Nonidet P-40, 0.1 μM $ZnCl_2$, 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 larger 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 preincubation 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′-agccagagaaatagaggagtctacagc-3′; mutant AP-1 core sequence GGAGTCT, 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 immunoprecipitations were performed using a 25-fold molar excess of peptide as described (39, 43).

Preparation of Recombinant Proteins—A full-length recombinant human p97/ERK3 cDNA sequence ("rhERK3") was cloned into the pET3C vector (Stratagene, La Jolla, CA). Thirty cycles of polymerase chain reaction were performed to amplify a linear *Bam*HI-*Eco*RI rhERK3 DNA fragment containing the full-length *ERK3* gene from plasmid

pGEX2T-ERK3, using forward primer 5′-agggtttccatatggcagagaaatttg-3′ and reverse primer 5′-tttgtgtgcatatgacatgccagttaa-3′. This method enabled in-frame insertion of the gene into the *Nde*I 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 endoproteinase 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 αc-Jun, 20 μg of αJunD (Santa Cruz Biotechnology), and 10 μl of αJunD (KG), all of which had been dialyzed 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 αc-Jun, 20 μg of αJunB, 20 μg of αJunD, 10 μl of αJunD (KG), 20 μg of αc-Fos, 20 μg of αFosB, 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 AP-1 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 M Phosphate Buffer, pH 8.2. Samples were incubated for 1 h with 400–500 μg of dialyzed NE at 4 °C with constant twirling and then washed 3 times with Dulbecco's phosphate-buffered saline (Life Technologies, Inc.), according to the manufacturer's instructions.

Protein Kinase Assays—Protein kinase assays were performed on immunoprecipitated and DNA affinity-purified proteins using a MAP kinase assay kit with the classic MAP kinase substrate myelin basic protein (MBP; Upstate Biotechnology, Inc.) with minor modifications of the manufacturer's protocol. Kinase assays of immunoprecipitated proteins bound to Dynal beads were performed directly after washing steps, without elution. For kinase assays of DNA-binding proteins, nuclear extracts were dialyzed, subjected to batchwise AP-1 DNA affinity chromatography with mutant AP-1 beads to eliminate nonspecifically associated material, and then subjected to batchwise DNA affinity chromatography with beads harboring a wild type AP-1 DNA sequence. Prior to assay, proteins bound to AP-1 DNA affinity beads were eluted with a 2.5-fold molar excess of wild type AP-1 oligo to maximize the specificity and the signal to noise ratio of the assay. Elution was performed in 30 μl of Kinase Assay Buffer (20 mM MOPS pH 7.2, 25 mM β-glycerophosphate (Sigma), 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol) containing 40 μg wt oligo at 4 °C for 30 min. Kinase assays were performed in a 50-μl reaction consisting of 10 μl of substrate (20 μg of MBP or 1 μg of recombinant c-Jun (Upstate Biotechnologies, Inc.), 1 μg of recombinant c-Fos, 10 μg of histone H1 (Roche Molecular Biochemicals), 10 μg of histone H3 (Roche Molecular Biochemicals), GST-CTD, or GST-JunD; 10 μl of [γ-^{32}P]ATP mixture (1 μl of 10 mCi, 6000 Ci/mmol; Amersham Pharmacia Biotech) diluted with proteins eluted in 9 μl of 75 μM $MgCl_2$ and 500 μM ATP in Assay Buffer), and 30 μl of Kinase Assay Buffer for 15 min at 30 °C.

In Vivo Metabolic Labeling—*In vivo* metabolic labeling of JB6 Cl30 7b cells with Translabel (ICN, Inc., Costa Mesa, CA) was performed as described (39). *In vivo* metabolic labeling of HT29 cells with Tran^{35}S-label was performed as described (39), except that labeling was done overnight in cells plated at 6×10^6 cells/150-mm tissue culture dish.

Two-dimensional Gels—Two-dimensional non-equilibrium pH gradient gel electrophoresis (NEPHGE 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 ^{35}S-metabolically labeled mouse epidermal JB6 Cl30 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 AP-1 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). αc-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 p36 (C; 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 ERP·AP-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 ranges 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

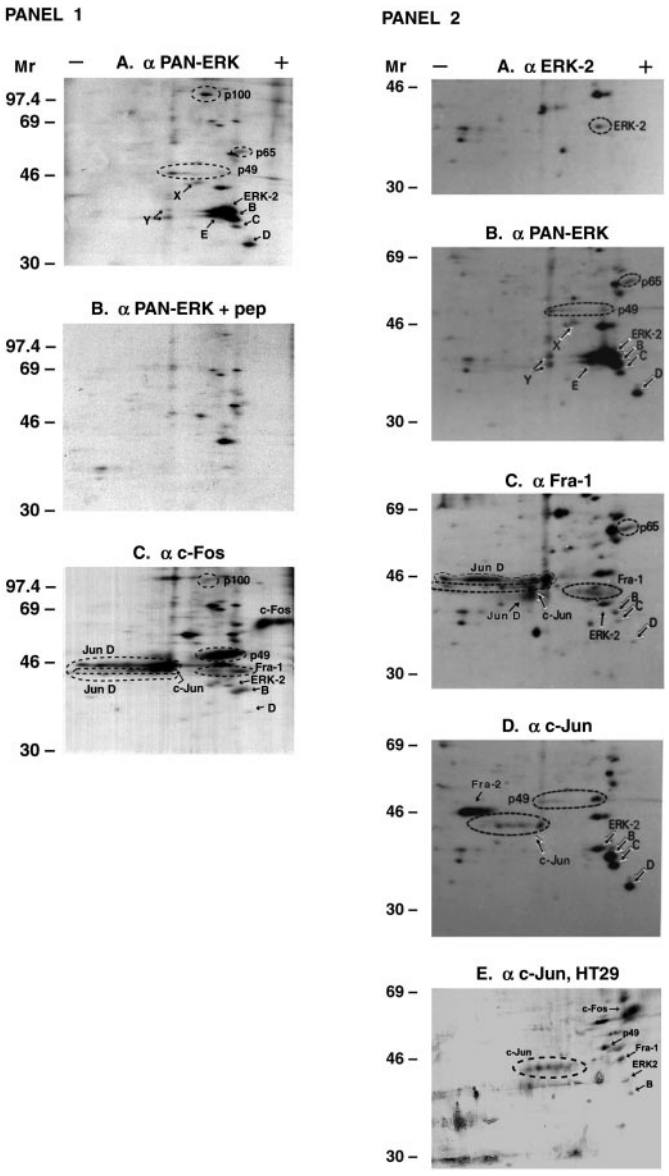

FIG. 1. **ERK2 and several other ERK-related proteins interact *in vivo* with multiple AP-1 transcription factor subunits.** Immunoprecipitations were performed with whole cell extracts from *in vivo* metabolically ^{35}S-labeled JB6 and HT29 cells with the indicated antibodies. *Panel 1, A–C*, JB6 cells. *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,* αERK1/2 1B3B9; *B*, αERK1-III (PAN anti-ERK αMAP kinase); *C,* αFra-1-(1–276); *D,* αc-Jun-(948-4); *E,* αc-Jun-(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 αp38 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.

ERK2 forms stable complexes *in vivo* with AP-1. However, the observation that multiple additional proteins immunologically related to MAP kinases bind AP-1 proteins *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.

Initial immunoblotting assays to test the efficacy of the binding assay were performed with AP-1 antibodies. AP-1 binding to the beads was demonstrated by immunoblotting of Laemmli SDS-PAGE gels with a mixture of antibodies against the AP-1 subunits c-Jun, JunB, and JunD (Fig. 2*A*). c-Jun and JunD were detected in association with AP-1 DNA on the affinity beads. Association of JunB was undetectable. Expression of c-Jun and JunD but not JunB proteins was also detected in unfractionated NE (not shown).

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. 2*A*). Specific binding to AP-1 DNA-binding sites from two different genes, the Gibbon ape leukemia virus long terminal repeat (GALV-LTR) and human

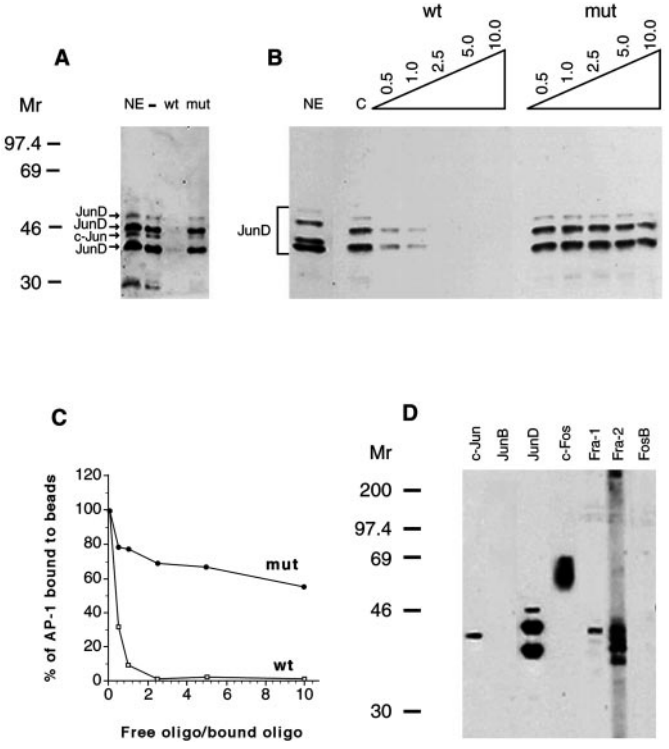

FIG. 2. **AP-1 dimers from HT29 adenocarcinoma cell nuclei interact specifically with affinity beads containing the AP-1 DNA and contain multiple Jun and Fos partners.** *A,* AP-1 dimers bind specifically to AP-1 affinity beads in the NAPSTER assay. HT29 cells were treated for 90 min with TPA, and NE 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 a mixture of specific antibodies against c-Jun, 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 analyzed 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. 2*D*). 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

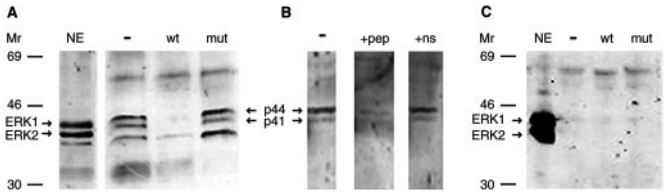

FIG. 3. **ERK-related proteins p41 and p44 associate along with AP-1 at the AP-1 DNA-binding site.** *A*, NAPSTER assay with αERK1-III. NE, nuclear extract directly loaded in SDS-PAGE. NE directly loaded in SDS-PAGE. Samples were eluted by boiling beads in SDS sample buffer, loaded in SDS-PAGE, and subjected 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 mg 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.*

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).

ERK-related Proteins p41 and p44 Associate with the AP-1 DNA-binding Site—To test the prediction that ERPs associate along with AP-1 DNA, we performed the NAPSTER assay and immunoblotted associated proteins with the PAN-ERK antibody αERK1-III. Three major proteins were detected with molecular masses of 41, 44, and 39 kDa. Of these three bands, two proteins, p41 and p44, associated specifically with the AP-1 DNA-binding site, since binding was specifically blocked when wild type AP-1 oligonucleotide was preincubated with nuclear extract, whereas mutant oligo had no effect (Fig. 3*A*). The 39-kDa protein bound to the beads in the presence of wild type AP-1 oligo, indicating that its binding to the AP-1 beads was nonspecific. Furthermore, as shown in Fig. 3*B*, p41 and p44 proteins are specifically recognized by the antigen-binding site within the PAN-ERK antibody, since preincubation of αERK1-III antibody with the ERK1-III peptide antigen but not a heterologous peptide antigen (from the ERK3 protein) blocked the association of p44 and p41 with the PAN-ERK antibody. By these criteria we refer to p44 and p41 as ERK-related proteins (ERPs). Therefore, by the NAPSTER assay, ERP proteins associate along with AP-1 proteins with the AP-1 DNA. Since the molecular weights of p41 and p44 are readily distinguishable from the class I ERPs, we designate them as class II ERPs. Although the class I ERPs were detectable with αERK1-III in Western blots as well as immunoprecipitations, they were not found in association with DNA, even when 1.5 mg of input NE was run in NAPSTER assays (not shown). Therefore class II but not class I ERPs associate with DNA along with AP-1. Furthermore, class I but not class II ERPs were detectable in association with AP-1 proteins in the absence of DNA.

ERK3-related Protein p97 Associates with AP-1 DNA-binding Site—We extended our search for potential ERPs that associate with the AP-1 DNA-binding site by performing the NAPSTER assay and immunoblotting with additional anti-MAPK antibodies. Among these was an antibody that recognizes human p97/ERK3. By these means a 97-kDa protein was detected in specific association with the AP-1 DNA-binding sequence (Figs. 4 and 5*B*). As for p41 and p44, p97 was detected bound to the AP-1 DNA beads if nuclear extracts were directly subjected to DNA affinity chromatography, or if they were preincubated

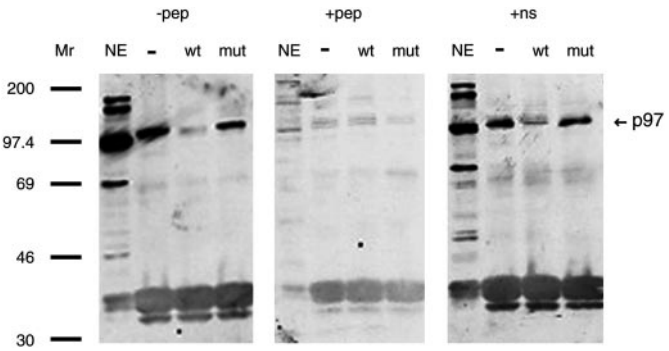

FIG. 4. **The ERK3-related protein p97 associates specifically with AP-1 DNA affinity beads.** The NAPSTER assay was performed using 300 μg of protein from NE per sample. Controls for specific detection of p97 protein by anti-ERK3 D23 antibody were performed by antibody preincubation with 25-fold molar excess of competitor peptide. *−Pep*, immunoblotting performed without preincubation of ERK3 D23 antibody with peptide. *+Pep*, preincubation with D23 peptide. *NS*, preincubation with I15 peptide (amino acids 353–367 of rat ERK3).

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. 3*A, 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 bound to the AP-1 beads, either in the presence or absence of preincubation with unbound oligo (Fig. 3*C*). Whereas αERK1-III detects ERK1 and ERK2 less strongly than the specific αERK1 antibody (compare nuclear extract samples from the two antibodies, *NE lanes* in Fig. 3, *A versus C*), only αERK1-III detects p41 and p44. These data demonstrate that p41 and p44 are not ERK1 or ERK2.

ERPs that Associate with the AP-1 DNA-binding Site Are New Species and Are Not Conventional MAP Kinases: MAP Kinase Proteins p38α, p38β, JNK1/SAPKγ, JNK2/SAPKα, JNK3/SAPKβ, ERK5/BMK1, and p38γ/ERK6/SAPK3 Are Not Detected in Association with AP-1·DNA Complexes—After finding that ERK1 and ERK2 do not associate with AP-1·DNA complexes, we investigated the possibility that other MAP kinase species specifically associate with the AP-1 DNA. Protein expression was assayed directly by Western analyses of whole NE, and DNA binding was assayed by the NAPSTER assay. Strong expression of p38α and/or p38β, JNK1/SAPKγ, JNK2/SAPKα, and/or JNK3/SAPKβ, and weak expression of ERK5/BMK1 proteins was detected in HT29 nuclear extracts. However, no specific association of these proteins with AP-1 DNA beads was detectable, even when 400 μg of input protein per sample were used in the NAPSTER assay (not shown). p38γ/ERK6/SAPK3 was not detectable in HT29 NE or bound to AP-1 DNA beads in NAPSTER assays even when as much as 1 mg of input NE was tested (not shown).

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. 5*A*) 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. 5*B*). However, whereas the other two ERK3 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 endoproteinase Glu-C ("V8 protease"). As shown in Fig. 5*E*, 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 αc-Jun, αc-Fos, and αJunD 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

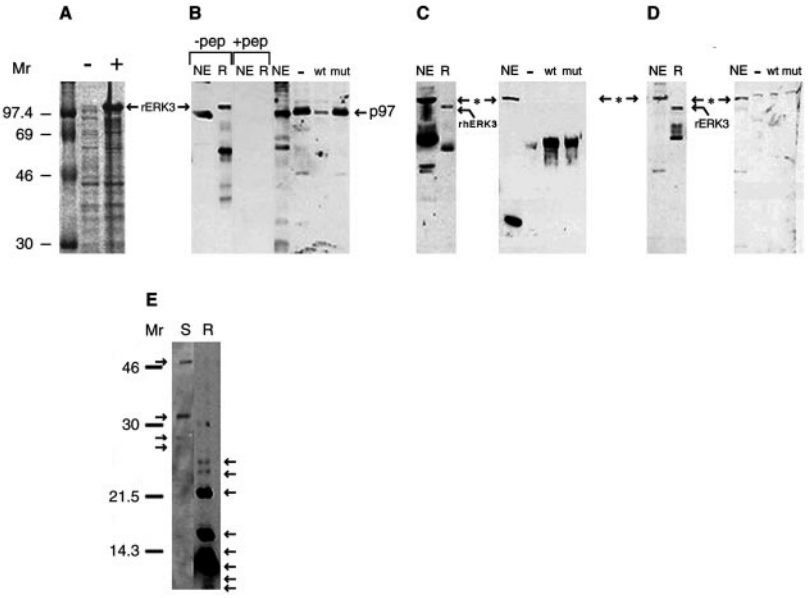

FIG. 5. **The AP-1 DNA-associated protein p97 is not human ERK-3.** *A,* expression of recombinant p97/ERK3 in bacteria. Extracts were made from *E. coli* transformed with the bacterial expression construct pET3C-ERK3 encoding full-length ERK3. Samples were run in SDS-PAGE and stained with Coomassie 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. αERK3 is against amino acids 303–325 of rat ERK3. *−pep*, without antibody preincubation with ERK3 D23 peptide; *+pep*, with preincubation with D23 peptide. *B–D*, samples were run in SDS-PAGE gels and immunoblotted. *R*, rhERK3, recombinant human p97/ERK3. *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 renatured 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. 7*A*). 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 αc-Jun plus αJunD depleted 80–90% of AP-1. Two rounds of depletion with these antibodies efficiently depleted Jun subunits to levels that were virtually undetectable (Fig. 7*B*). Two rounds of AP-1 immunodepletion also eliminated association of p41 and p44 ERPs as evidenced

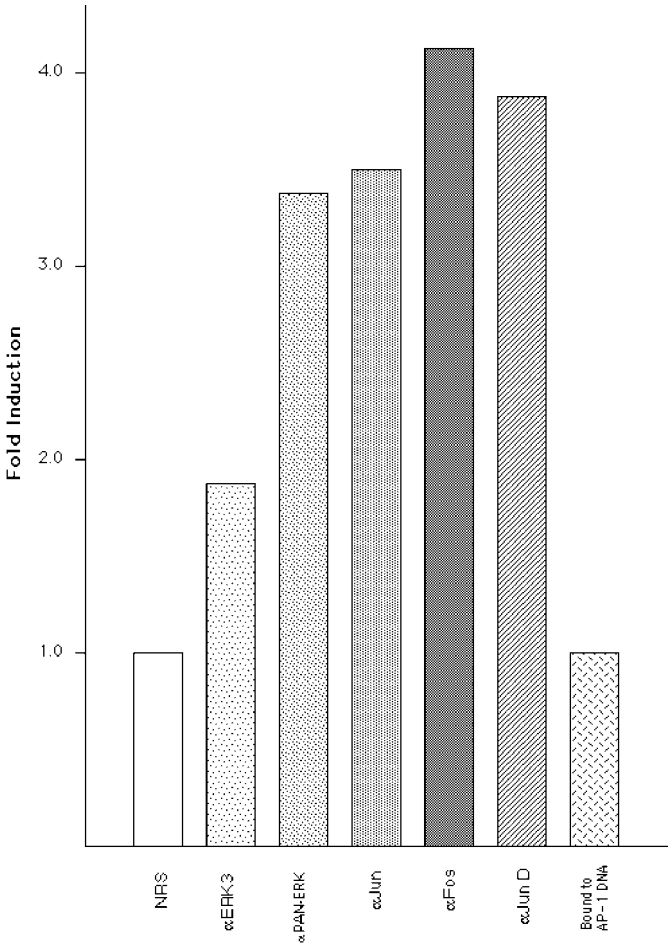

FIG. 6. **AP-1 has stably associated kinase activity when not bound to AP-1 DNA.** Immunoprecipitations with 750 μg of whole cell extract or dialyzed HT29 NE were performed with antibodies against MAP kinases or AP-1, and *in vitro* kinase assays were performed with MBP as described under "Experimental Procedures." Specificity controls consisted of peptide antibody samples preincubated with antigenic competitor peptides prior to immunoprecipitation. Controls for antibodies for which no peptide competitor was available consisted of preincubations with normal rabbit serum (*NRS*), followed by protein A precipitations. AP-1 DNA affinity chromatography with 600 μg of NE was performed by preincubation with mutant AP-1 DNA beads followed by wild type beads as described above. Kinase activity was quantitated by liquid scintillation counting of incorporated ^{32}P into MBP. For immunoprecipitates, quantitation is expressed as fold induction relative to newborn calf serum (for αc-Jun and αc-Fos) or to immunoprecipitates with antibody preincubated with antigenic peptide (for αERK3, αPAN-ERK, and αJunD). For samples isolated by DNA affinity chromatography, fold induction is expressed relative to NAPSTER isolated samples for which NE was preincubated with excess free wild type AP-1 oligo. Samples from *left* to *right*: NRS, nonimmune rabbit serum; αERK3 D23; αPAN-ERK (αERK1-III); αc-Jun-(948-4); αc-Fos (Upstate Biotechnologies, Inc.); αJunD SC; proteins associated with AP-1 DNA.

in Western immunoblotting with αERK1-III of NAPSTER-isolated material (Fig. 7*B*). 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. 7*B*). 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. 7*C*). 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 coimmunoprecipitation 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 coimmunoprecipitation 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-

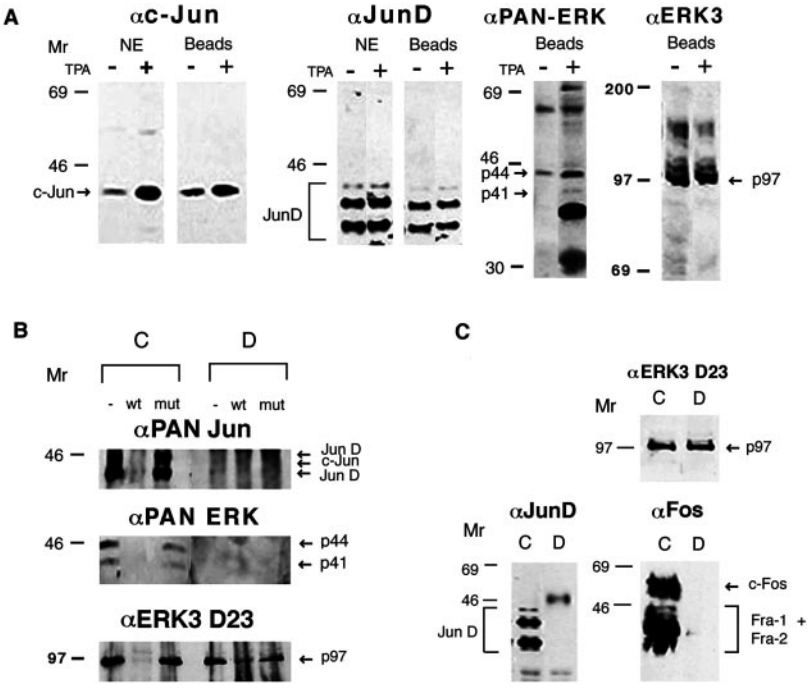

FIG. 7. *A,* association of c-Jun, p41, and p44 with AP-1 DNA is TPA-inducible, whereas association of JunD and p97 is not. HT29 cells were treated with TPA (+) or with Me_2SO control (−) for 90 min prior to harvest and NE preparation. 100 μg of NE (for c-Jun and JunD detection), 3.3 mg of NE (for p41 and p44 detection), or 1.1 mg of NE (for p97 detection) were subjected to AP-1 DNA chromatography followed by Western analyses with αc-Jun, αJunD, αPAN-ERK, and αERK3 D23 antibodies. c-Jun, JunD, p41, p44, and p97 are indicated by *arrows* or *brackets. B,* association of p44 and p41 ERPs with DNA is dependent on AP-1 proteins. Nuclear extracts underwent mock immunodepletion (*C*, control with no antibody) or two rounds of immunodepletion (*D*) with a mixture of αc-Jun and αJunD antibodies, prior to the NAPSTER assay. Western analyses were performed with immunodepleted NAPSTER assay samples: *top panel*, αJuns; *middle panel*, αERK1-III; *bottom panel*, αERK3 D23. 120 μg per NAPSTER assay sample of NE with respect to protein was used for αPAN Jun, and 1.2 mg per sample was used for αERK1-III and αERK3 D23. JunD, c-Jun, p41, p44, and p97 are indicated with *arrows.* Similar results were obtained in two independent experiments. *C,* association of p97 ERP is independent of AP-1 proteins. Nuclear extracts underwent mock immunodepletion (C) or three rounds of immunodepletion (D) with a mixture of antibodies against all seven AP-1, and were subsequently subjected to AP-1 DNA affinity chromatography, loaded in SDS-PAGE, and subjected to Western immunoblotting with αERK3 D23 (*upper panel*), αJunD (*lower left panel*), or αFos antibody that recognizes multiple Fos family proteins (*lower panel*). 180 μg of NE per sample with respect to protein was used for DNA affinity chromatography for αPAN Jun, and 1.8 mg per sample was used for αERK1-III and αERK3 D23. p97 and c-Fos are indicated by *arrows*, and JunD, Fra-1, and Fra-2 are indicated by *brackets.*

TABLE I
Four classes of ERPs

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

[a] N.O., not observed.

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 proteins 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, 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. 3*C*), 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.[2] 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.[2] 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 *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-1 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-ERK 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-ERK 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 DNA. 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: αJunD (KG) and GST-JunD from Dr. K. Gardner (National Institutes of Health, Bethesda); αJun-(948-4) from Dr. D. Gillespie (Beatson Institute for Cancer Research, Glasgow, Scotland, UK); αFra-1-(1–276) from Dr. R. Bravo (amino acids 1–276; Bristol Myers-Squibb Co.); αERK3 "C" from Dr. M. Cobb (University of Texas Southwestern Medical Center, Dallas); αhERK3 "F" and hERK3 expression construct from Dr. J Flier (Charles A. Dana Research Institute, Boston); pGCTD plasmid from Dr. W.S. Dynan (Indiana University School of Medicine, Indianapolis); purified recombinant c-Fos from T. Kerppola (University of Michigan, Ann Arbor, MI). We thank Dr. Douglas Ferris for technical assistance and Drs. Kenneth Ramos, Julian Liebowitz, David Peterson, and Michael Kladde for helpful discussions and comments on the manuscript.

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[3] N V. Kumar and L. R. Bernstein, unpublished data.

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

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