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Isolation of an AP-1 Repressor by a Novel Method for Detecting Protein-Protein Interactions

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Transcription factor AP-1 transduces environmental signals to the transcriptional machinery. To ensure a quick response yet maintain tight control over AP-1 target genes, AP-1 activity is likely to be negatively regulated in nonstimulated cells. To identify proteins that interact with the Jun subunits of AP-1 and repress its activity, we developed a novel screen for detecting protein-protein interactions that is not based on a transcriptional readout. In this system, the mammalian guanyl nucleotide exchange factor (GEF) Sos is recruited to the *Saccharomyces cerevisiae* plasma membrane harboring a temperature-sensitive Ras GEF, Cdc25-2, allowing growth at the nonpermissive temperature. Using the Sos recruitment system, we identified new c-Jun-interacting proteins. One of these, JDP2, heterodimerizes with c-Jun in nonstimulated cells and represses AP-1-mediated activation.

Transcription factor AP-1 is a collection of dimers composed of Jun (c-Jun, JunB, and JunD), Fos (c-Fos, FosB, Fra1, and Fra2), or ATF (ATF2, ATF3/LRF1, and B-ATF) subunits that interact through the leucine zipper motif and bind DNA through a basic region (5, 44). The Jun proteins are most versatile and capable of forming Jun-Jun, Jun-Fos, and Jun-ATF dimers (15, 18, 19, 28, 31, 40, 45). Jun-Jun and Jun-Fos dimers bind AP-1 sites (also known as TPA-responsive elements [TREs]), while Jun-ATF dimers prefer the related ATF binding site (also known as the cyclic AMP-responsive element [CRE] [18]). A number of other bZIP proteins that can heterodimerize with Jun, Fos, and ATFs have also been characterized. Among these, Maf proteins (v-Maf and c-Maf [34]) and the neural retina-specific gene product Nrl (43) can form heterodimers with c-Jun or c-Fos which bind to TRE, CRE, and an asymmetric DNA sequence consisting of half each of consensus of Maf and TRE (27). A group of Maf-related proteins, MafB, MafF, MafG, and MafK, which are expressed in a wide variety of tissues, have also been shown to form heterodimers with Fos but not Jun (17, 25, 26). These combinatorial interactions expand the diversity of genes targeted by AP-1 and their regulation by extracellular stimuli. The general function of AP-1 is to convert environmental stimuli to changes in target gene transcription (24). To do so, AP-1 must respond quickly to extracellular stimuli yet be maintained at low basal levels in nonstimulated cells. Most cell types exhibit substantial AP-1 DNA binding but a low level of transcriptional activity prior to stimulation (2). Following a variety of extracellular stimuli, AP-1 transcriptional activity is rapidly and substantially induced while DNA binding activity is only modestly increased (24). Although the mechanisms of induction of AP-1 activity in stimulated cells are relatively well understood (24), it is not clear what maintains the low level of AP-1 transcriptional activity in nonstimulated cells. Dimerization with inhibitory subunits as a suppression mechanism has been described for several transcription factors (7, 10, 32, 33, 35, 38, 39). For example, it was recently shown that expression of MafB in erythroid cells results in the repression of the Ets-1 transcriptional activity through protein-protein interaction and inhibition of cell differentiation (39). Dominant negative Jun or Fos derivatives have also been engineered to inhibit AP-1 biological activities (11, 30).

To isolate natural inhibitory subunits of AP-1, we developed a new genetic screening system that detects protein-protein interactions. This system is designated the SOS recruitment system (SRS), and unlike the conventional two-hybrid system (16), it is not based on a transcriptional readout. The conventional two-hybrid system, although very powerful, is based on a transcriptional readout and may not be suitable for identifying transcriptional repressors (1). Furthermore, proteins that activate transcription cannot be used in the conventional two-hybrid system, unless their transcriptional activity is attenuated. The SRS circumvents this problem. We used the SRS successfully to isolate two novel proteins, JDP1 and JDP2, which interact specifically with c-Jun. We show that JDP2 is associated with c-Jun in the cell and strongly inhibits c-Jun transcriptional activity.

MATERIALS AND METHODS

Plasmids and constructs. DNA fragments to be inserted in frame with sequences encoding either the hSos catalytic domain or the v-Src myristoylation signal were generated by PCR. The following pADNS expression vectors were used: pADNS empty expression vector (ADNS) and pADNS expression vectors that encode the DNA binding domain (DBD) of c-Jun (amino acids 249 to 331) fused to h5′Sos (amino acids 1 to 1066) at either its amino (JZ-5′Sos) or carboxy (5′Sos-JZ) terminus; the c-Jun activation domain (AD) (amino acids 1 to 223) fused to 5′Sos (JA-5′Sos); the DBD and carboxy-terminal AD of c-Fos (amino acids 133 to 239) fused to full-length hSos (Fos-Sos); pYes2 empty expression vector (Yes2); pYes2 expression vectors that encode myristoylated c-Jun DBD (amino acids 249 to 331, M-Jun); the DBD and carboxy-terminal AD of c-Fos (HOB1) fused to Src myristoylation signals (M-Fos; hc-Fos amino acids 133 to 239); and ADNS and Yes2 expression vectors encoding p110β (amino acids 31 to 150) fused to 5′Sos (p110-5′Sos) and p85 (amino acids 427 to 613) fused to the v-Src myristoylation signal (M-p85) and full-length and truncated (amino acids 1 to 1066) Sos fused to Ras farnesylation signal (SosF or 5′SosF).

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Yeast growth and manipulations. Conventional yeast transfection and manipulation protocols (6) were used. Cells were plated on either glucose minimal medium containing the relevant amino acids, 2% glucose, 0.5% NH_4SO_4, 0.17% yeast extract, and 4% agar or galactose medium containing 3% galactose, 2% raffinose, and 2% glycerol instead of glucose. Replica plating was performed with a disposable ACCUTRAN replica plater (Schleicher & Schull, Inc.).

Library construction and screening. Double-stranded cDNA was generated from 7 μg of poly(A^+) RNA isolated from rat GC pituitary cells by using a random primed cDNA synthesis kit (Stratagene Inc.). The cDNAs were digested with *Eco*RI and *Xho*I and inserted into pYes2 containing the v-Src myristoylation sequence and a consensus translation initiation sequence (described in reference 6), predigested with the appropriate restriction enzymes. The total library contains approximately 2×10^6 independent clones. *cdc25-2* cells were cotransfected with 3 μg of pADNS-5′Sos-JZ expression vector and 3 μg of pYes2-cDNA library plasmids (resulting in ~10,000 transformants per plate) and grown for 4 days at 25°C on selectable minimal glucose plates. Subsequently, colonies were replica plated onto minimal galactose plates and grown at 36°C for 2 to 3 days. A total of 64 colonies were isolated and tested for galactose-dependent growth at 36°C. Plasmids were extracted from 19 colonies and transformed into *Escherichia coli* to be further analyzed by DNA sequencing and retransformation of *cdc25-2* cells together with either p5′Sos-JZ, pJA-5′Sos, or pADNS to test the specificity of interaction and dependence on the c-Jun DBD.

Isolation of full-length cDNAs and sequence analysis. The inserts of clones 8 and 11 were used to rescreen the cDNA library to obtain longer cDNAs. PCR amplification of the cDNA 5′ end was performed with a rat spleen cDNA (Clontech, Inc.), as specified by the manufacturer, to isolate full-length JDP2 cDNA. DNA sequencing was performed with the Sequenase version 2 kit (United States Biochemical, Inc.). Phylogenetic comparison was done with the PILEUP program (University of Wisconsin Genetics Computer Group) by using progressive pairwise alignments. The protein sequences of bZIP proteins were obtained from the Swiss Protein and GenBank databases.

Protein analysis. The coding regions of JDP1 and JDP2 were fused in frame to six histidines (36), and nuclear extracts was prepared as described by Schreiber et al. (37). Protein extracts (50 μg/lane) were resolved on a sodium dodecyl sulfate (SDS)–13% polyacrylamide gel, blotted onto a nitrocellulose membrane probed with JDP1 or JDP2 antibodies, and developed with a chemiluminescence reaction kit (Amersham Inc.).

In vitro translation and DNA binding analysis. The different plasmids (1 μg each) were used to program a coupled SP6/T7 transcription-translation rabbit reticulocyte system as specified by the manufacturer (Promega, Inc.). The different lysates were incubated in binding buffer [25 mM HEPES (pH 7.9), 150 mM KCl, 2% glycerol, 5 mM dithiothreitol, 1 μg of poly(dI-dC)] for 5 min at room temperature, and ^{32}P-labeled *jun*-TRE or *col*-TRE oligonucleotide probes were added (15,000 cpm). After a 20-min incubation, the samples were resolved on a 6% nondenaturing polyacrylamide gel in 0.5× TBE buffer and autoradiographed.

Cell culture transfection experiments. NIH 3T3 cells were cotransfected by the calcium phosphate coprecipitation method with *jun*-TRE luciferase reporter plasmids (1 μg) together with the following expression plasmids: vector alone (3 μg), RSV–c-Jun (1 μg), RSV–c-Jun (1 μg each) with expression plasmids pCINeo (Promega Inc.) encoding either JDP1 or JDP2, a murine sarcoma virus (MSV) expression plasmid encoding c-Fos, or a simian virus 40 expression plasmid encoding Fra2 (2 μg each). The cells were harvested 24 h after transfection, and luciferase activity was determined. The results represent the mean of three independent experiments. Nuclear extract derived from cells transfected with the corresponding plasmids was used to confirm the relative levels of c-Jun protein following coexpression with JDP1, JDP2, Fra, or c-Fos proteins by using the 3×HA–c-Jun expression vector (containing three copies of the hemagglutinin epitope tag, HA, fused to c-Jun) with anti-HA antibodies.

Immunoprecipitations. Immunoprecipitation was performed with prebound antiserum to protein A-Sepharose beads in phosphate-buffered saline containing 0.5% Nonidet P-40 and 1% sodium deoxycholate (buffer A). Nuclear extract was added, and the mixture was incubated for 2 to 4 h at 4°C. The beads were washed three times with buffer A, SDS sample buffer was added, and the protein was resolved on an SDS–10 or 13% polyacrylamide gel.

TRE pulldown assay. Nuclear extracts (100 μg) of serum-deprived and serum-stimulated NIH 3T3 cells were incubated with 50 μg of poly(dI-dC) and 50 μg of either nonspecific DNA competitor (GRE) or specific competitor (Jun-TRE) in 1× gel shift binding buffer for 30 min at room temperature. To this was added 30 μl of AP-1–Sepharose affinity resin, and the mixture was stirred at room temperature for 90 min. Beads were collected by centrifugation and washed three times with gel shift buffer containing 200 mM NaCl. SDS sample buffer was added to the beads, and the proteins were resolved on an SDS–12% polyacrylamide gel and then subjected to Western blot analysis with antibodies described in the legend to Fig. 7D.

Nucleotide sequence accession numbers. The accession numbers for JDP1 and JDP2 are U53450 and U53449, respectively.

RESULTS

SRS. We took advantage of the finding that the human Ras guanyl nucleotide exchange factor (GEF), hSos, can substitute for the *Saccharomyces cerevisiae* Ras GEF, Cdc25, to allow cell survival and proliferation (6). The hSos-mediated rescue of a temperature sensitive *cdc25-2* allele depends on its recruitment to the plasma membrane, mimicking the effect of growth factor receptor activation (Fig. 1A and B). In theory, this recruitment could also be mediated by interaction between two hybrid proteins (Fig. 1C and D) and thus could serve as a basis for a novel method for detecting protein-protein interactions, the SRS. A series of yeast expression plasmids based on the pADNS plasmid (12) were constructed encoding chimeras of C-terminally truncated hSos and fragments of other proteins known to engage in heterooligomerization. The C-terminal truncation which removes the Grb2 binding region (29) results in high Sos activity and still depends on membrane localization (6). A second series of plasmids (based on pYes2) code for membrane-targeted versions of the interacting partners.

Interaction of two nuclear proteins, c-Jun and c-Fos, in SRS. As an example of the interaction of nuclear proteins, we first tested whether c-Jun and c-Fos heterodimers (5) can recruit hSos to the membrane and activate Ras. The c-Jun DBD (the leucine zipper and basic region) was fused in frame to either the amino (JZ-5′Sos) or carboxy (5′Sos-JZ) terminus of hSos. A fragment of c-Fos, containing its basic region, leucine zipper, and C-terminal AD (4, 13, 42), was fused to the Src myristoylation signal (M-Fos [Fig. 2A]). As a control, the c-Jun activation domain (AD) (4) was fused to the amino terminus of hSos (JA-5′Sos [Fig. 2A]). Pairs of plasmids were introduced into *cdc25-2* cells, and transformants were selected at 25°C, replica plated, and transferred to 36°C. Only cells containing pJZ-5′Sos plus pM-Fos, but not pM-Fos plus pADNS or pYes2 plus pJZ-5′Sos, grew at 36°C (Fig. 2B). Similar results were obtained when the c-Jun DBD was fused to the carboxy terminus of hSos (5′Sos-JZ). Cells containing M-Fos and c-Jun AD fused to hSos (JA-5′Sos [Fig. 2A]) did not grow at 36°C (Fig. 2B), consistent with the lack of interaction between this part of c-Jun and c-Fos (4). We also fused the c-Fos fragment to full-length hSos (Fos-Sos) and tested its interaction with c-Jun DBD fused to the myristoylation signal (M-JZ [Fig. 2A]). While pM-JZ plus pADNS or pFos-Sos plus pYes2 did not suppress the *cdc25-2* phenotype, transformation with pM-JZ plus pFos-Sos did (Fig. 2C). Thus, the interaction between c-Jun and c-Fos, which has been difficult to study by the conventional two-hybrid system because of their potent activation domains (24a, 41), can be detected in SRS. The presence of a bona fide or incidental activation domain on the bait protein in the conventional two-hybrid system results in high background that reduces the sensitivity of the system and interferes with isolation of novel proteins that interact with that particular bait (1). Our results also suggest that the presence of nuclear localization sequences (14) in the DBDs of c-Fos and c-Jun does not interfere with membrane localization, probably because the myristoylation signal is dominant (22).

Interaction of two cytoplasmic proteins, p110 and p85, in SRS. We also examined whether the interaction of two cytoplasmic proteins, the subunits of phosphatidylinositol-3-phosphate kinase (23), can be detected by SRS. We fused p110β (20, 21) to hSos (p110-5′Sos) and p85 to the myristoylation signal (M-p85) (Fig. 3A). Cells expressing either p110-5′Sos or M-p85 did not grow at 36°C, but cells expressing both proteins did (Fig. 3B and C). p110-5′Sos does not interact with either M-JZ or M-Fos (Fig. 3C). Thus, pairs of specifically interacting cytoplasmic or nuclear proteins can recruit Sos to the mem-

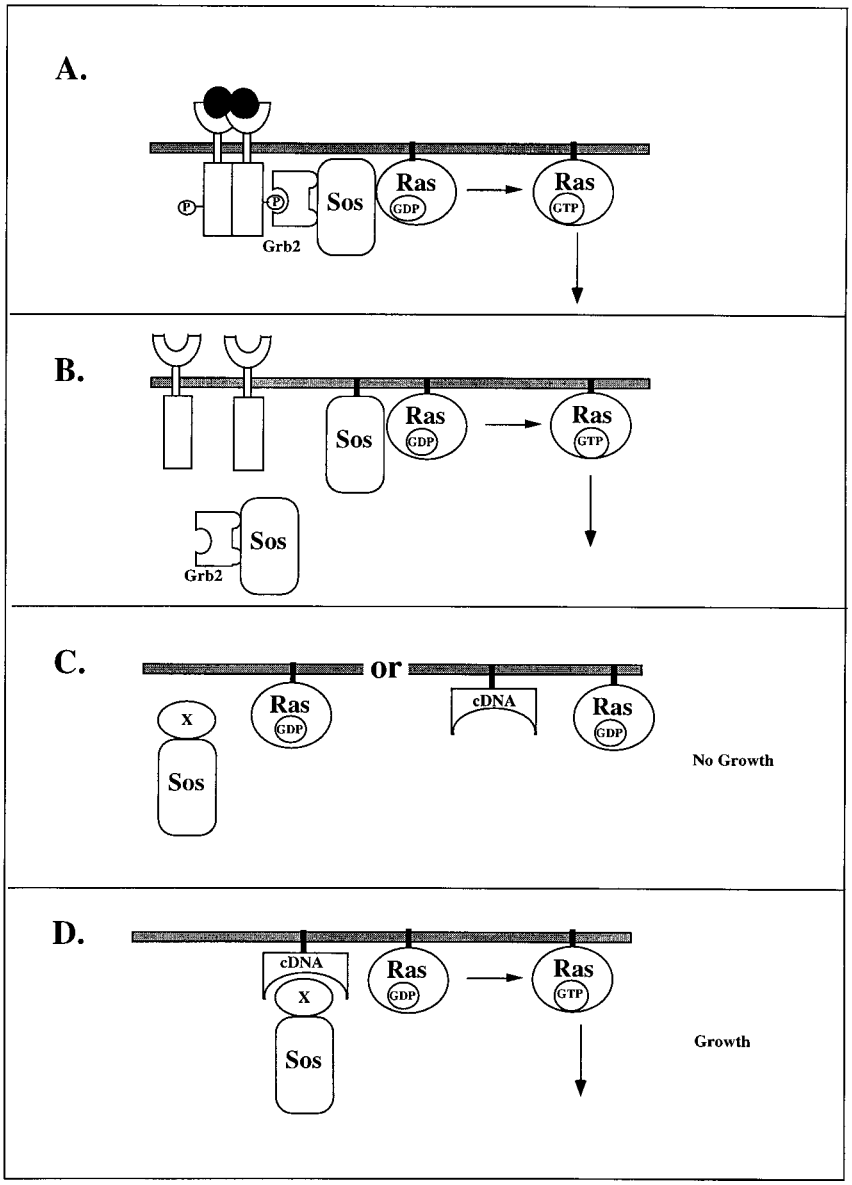

FIG. 1. Ras activation by Sos via growth factor receptors (A) or constitutive targeting to the plasma membrane (B) and the Sos recruitment system (C and D). (A) Sos is complexed with Grb2 in nonstimulated cells. Growth factor receptor activation provides phosphotyrosine recognition sites for Grb2, resulting in recruitment of the Grb2-Sos complex to the receptor and the plasma membrane, where its substrate, Ras, is located. (B) When Sos is fused to myristoylation or farnesylation signals, it is concentrated in the plasma membrane, leading to ligand-independent Ras activation. (C and D) In cells that express a chimera of Sos-X (where X is a target protein) along with a membrane-localized version of a protein that interacts with X (cDNA), the interaction between X and its partner recruits Sos to the plasma membrane, resulting in Ras activation.

brane and activate Ras. This result further supports the notion that plasma membrane translocation of Sos is a critical event in growth factor signaling (6).

Isolation of c-Jun interacting proteins. c-Jun is the major component of AP-1 in nonstimulated cells (5, 24). In an effort to isolate c-Jun-interacting proteins, we used its DBD to screen an expression library of rat pituitary cDNAs that was fused to the Src myristoylation signal. The expression of fusion genes was controlled by a galactose-inducible promoter, thus enabling a direct identification of cDNAs responsible for suppression of the *cdc25-2* phenotype. Approximately 2×10^5 transformants containing library plasmids and the 5′Sos-JZ target were grown at 25°C, replica plated onto galactose plates, and transferred to 36°C. Cells that grew at 36°C were isolated and tested for growth in the presence of either glucose or galactose. Library plasmids were isolated from clones that exhibited galactose-dependent growth at 36°C and retransformed into *cdc25-2* cells with either the original target (5′Sos-JZ), a nonrelevant target (JA-5′Sos), or pADNS. Twelve plasmids suppressed the *cdc25-2* phenotype only in the presence of 5′Sos-JZ (Fig. 4A). Nine clones encoded Fra-2, and one encoded FosB, both of which heterodimerize with c-Jun (31, 45). Two additional clones encoded proteins that specifically interacted with c-Jun DBD, and based on their sequences, they are novel AP-1 proteins. Clones which did not exhibit Jun-dependent growth encoded members of the Ras family (data not shown). Overexpression of mammalian Ras proteins in yeast bypasses *CDC25* (9).

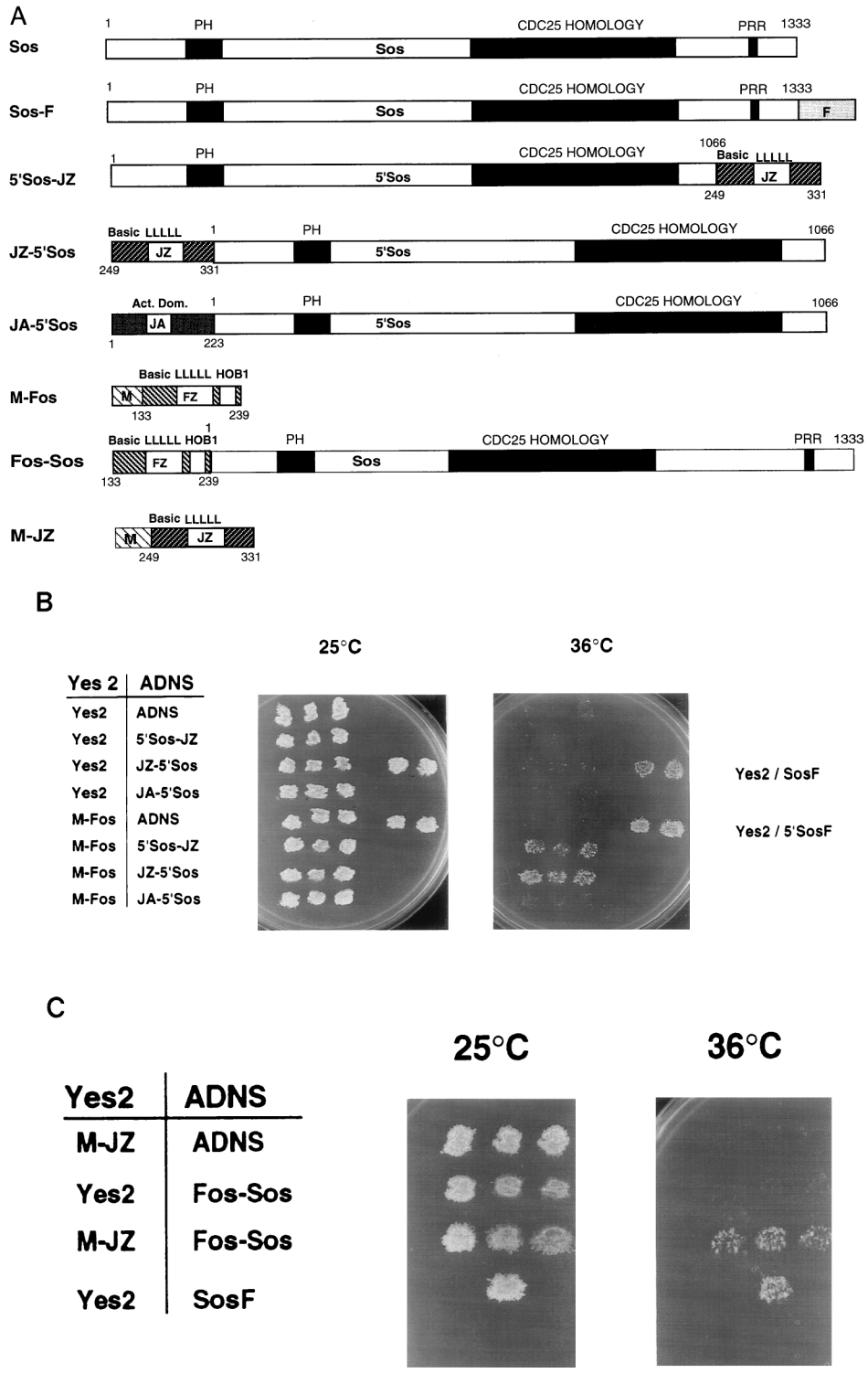

FIG. 2. Complementation of the *cdc25-2* mutation through Jun-Fos dimerization. (A) The various constructs used in these experiments. PH, pleckstrin homology; CDC25, Sos catalytic domain; PRR, proline-rich region; F, farnesylation signal; M, myristoylation signal; Basic, basic regions of c-Jun and c-Fos; LLLLL, leucine zipper; HOB1, c-Fos activation domain. (B and C) *cdc25-2* cells (*MAT*α *ura3 lys2 leu2 trp1 cdc25-2 his3Δ200 ade101 GAL*$^+$) were transformed with the indicated plasmids and plated on glucose minimal medium supplemented with the appropriate amino acids and bases. Three independent transformants (only two for pYes2-SosF and pYes2-5′SosF) were grown at 25°C (left panels), replica plated onto appropriately supplemented galactose minimal plates, and grown at 36°C (right panels). Only transformants expressing both c-Jun and c-Fos bZIP-containing chimeras grow efficiently at 36°C, as well as the positive control Sos-F- and 5′SosF-transformed cells.

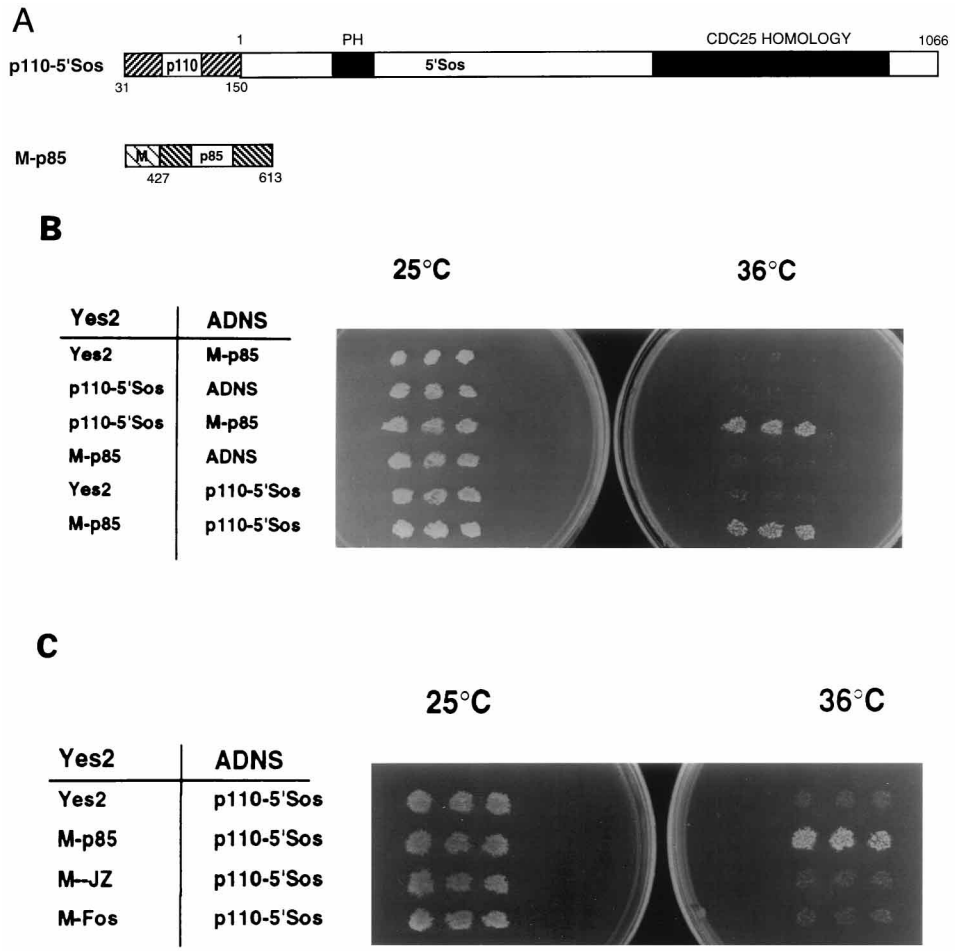

FIG. 3. Complementation of *cdc25-2* through p110-p85 interaction. (A) Constructs used in these experiments. Abbreviations are as described in the legend to Fig. 2. (B and C) Cells were transformed and replica plated as described in the legend to Fig. 2. (Left panel) Cells grown at 25°C; (right panel) cells grown at 36°C. Only transformants expressing both p110- and p85-containing chimeras grow efficiently at 36°C.

The novel c-Jun-dependent clones were used as probes to screen the same library, and longer cDNAs were isolated (Fig. 4B and C). Sequence comparison (Fig. 4D) shows that clones 8 and 11 are most similar to B-ATF and LRF1/ATF3, respectively (50 and 72% identity, respectively) (15, 19). No similarities to other AP-1 proteins were detected outside the DBDs. The two polypeptides were named Jun dimerization protein 1 (JDP1) (clone 8) and JDP2 (clone 11). Northern blot analysis revealed that JDP1 and JDP2 transcripts are present in all tissues and cells analyzed (data not shown). Polyclonal antibodies generated against recombinant JDP1 or JDP2 proteins reacted with polypeptides of the same size in various murine cell lines (Fig. 5A).

The DNA binding activities of JDP1 and JDP2, as well as their interaction with c-Jun, were examined by a mobility shift assay with the AP-1 site of the c-*jun* promoter (*jun*-TRE) as a probe (3). JDP1 did not bind *jun*-TRE by itself, but cotranslation with c-Jun resulted in the appearance of a binding activity whose electrophoretic mobility is different from the one attributed to c-Jun homodimers (Fig. 5B). JDP2 bound the *jun*-TRE by itself and, unlike JDP1, enhanced the binding of c-Jun to this element. JDP–c-Jun heterodimers also bound to the consensus TRE of the collagenase promoter (data not shown). The sequence specificity of the different complexes was demonstrated by competition experiments, and the presence of HA-tagged c-Jun in the two heterodimers was examined by anti-HA antibody supershift experiments (data not shown). The in vitro interaction of JDP2 with JunB and JunD was also examined (Fig. 5C). JDP2 formed heterodimeric complexes with both proteins. The stability of the JDP2-JunB and JDP2-JunD dimers is comparable to that of the c-Jun–JDP2 dimers (Fig. 5C). These results demonstrate that JDP1 and JDP2 are indeed bona fide members of the AP-1 group that heterodimerize with c-Jun or other Jun proteins.

JDP2 inhibits c-Jun and AP-1 transcriptional activation. To examine the role of JDP1 and JDP2 in regulating the transcriptional activity of AP-1, NIH 3T3 cells were cotransfected with a *jun*-luciferase (Luc) reporter (3) and various combinations of c-Jun, JDP1, JDP2, Fra2, and c-Fos expression vectors (Fig. 6A). As expected c-Fos and Fra2 enhanced the ability of c-Jun to activate *jun*-Luc while JDP1 had only a marginal effect on c-Jun activity (Fig. 6A). Coexpression of JDP2, however, repressed the ability of c-Jun to activate the *jun*-Luc reporter. Immunoblot analysis indicated that these effects were not due to changes in c-Jun expression. JDP2 also repressed reporter activation by a combination of c-Jun and c-Fos (Fig. 6B) but had no effect on the transcriptional activity of either GAL4DBD–c-JunAD chimera or transcription factor E2A (data not shown). Comparable amounts of c-Jun and JDP2 were coprecipitated by either JDP2 or c-Jun antibodies from

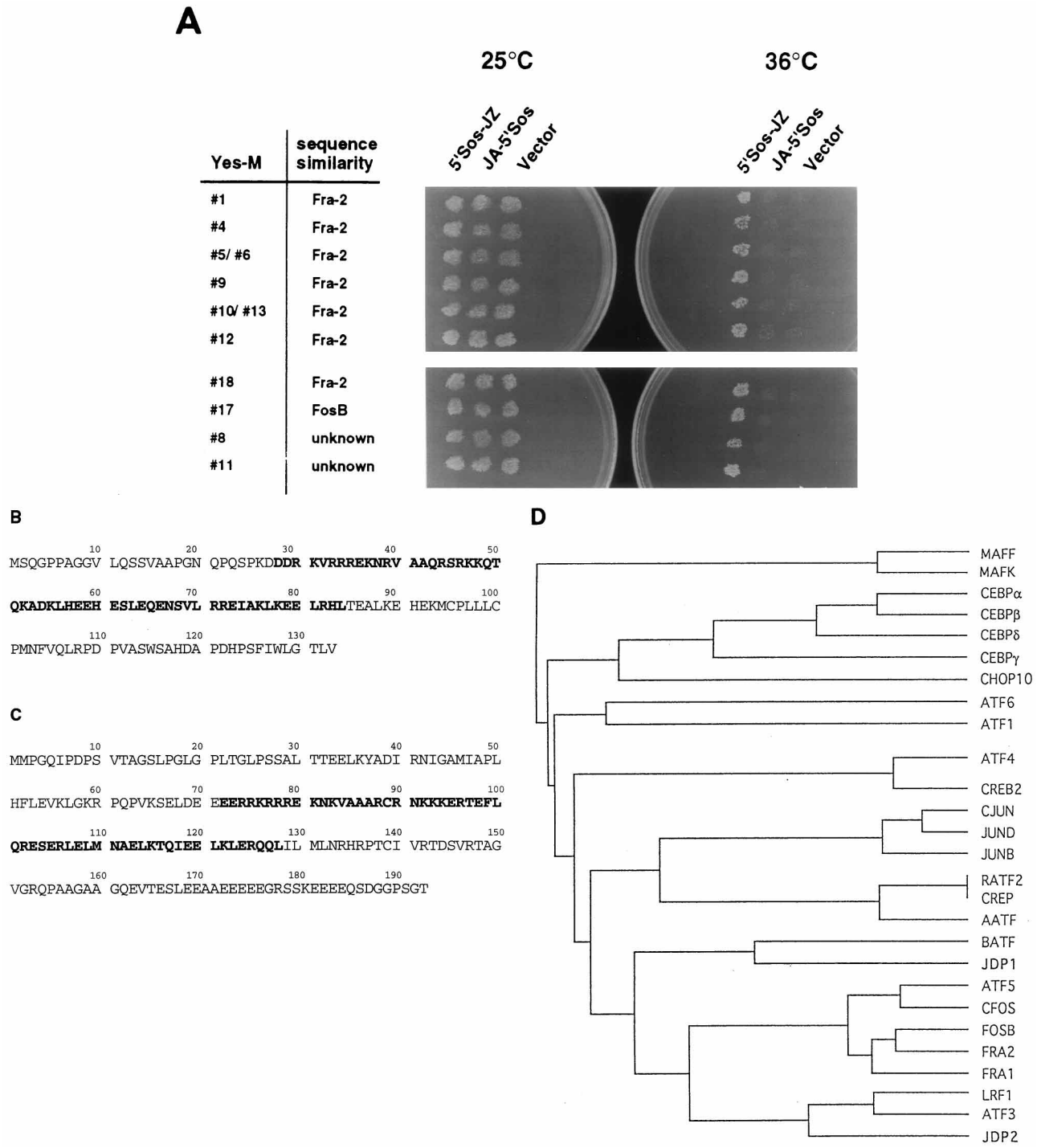

FIG. 4. Isolation of c-Jun DBD-interacting proteins. (A) A rat pituitary cDNA library in which the cDNAs are fused to a 5′ myristoylation signal was cotransformed into *cdc25-2* cells along with the 5′Sos-JZ expression vector. Colonies expressing both plasmids were selected at 25°C (approximately 2×10^5 transformants), and after replica plating and incubation at 36°C, the library plasmids were isolated from colonies that grew. These plasmids were retransformed into *cdc25-2* cells together with either pJZ-5′Sos, pJA-5′Sos, or pADNS. Individual colonies were isolated and grown at 25°C (left panel) and replica plated onto galactose plates and grown at 36°C (right panel). (B and C) Complete polypeptide sequences of JDP1 and JDP2. The basic regions and leucine zippers are highlighted. (D) A dendrogram comparing the DBD of JDP1 and JDP2 with those of other basic region-leucine zipper proteins.

extracts of cells coexpressing c-Jun and JDP2. This indicates that JDP2 quantitatively associates with c-Jun (Fig. 6C). These antibodies were not cross-reactive, and the interaction between c-Jun and JDP2 was disrupted by inclusion of 0.1% SDS in the immunoprecipitation buffer (data not shown).

To examine whether endogenous c-Jun and JDP2 interact, nuclear extracts of PC12 and NIH 3T3 cells were incubated with either preimmune serum, c-Jun, or JDP2 antibodies. In both cases, similar amounts of c-Jun were precipitated by either c-Jun or JDP2 antibodies (Fig. 7A). Since the closest

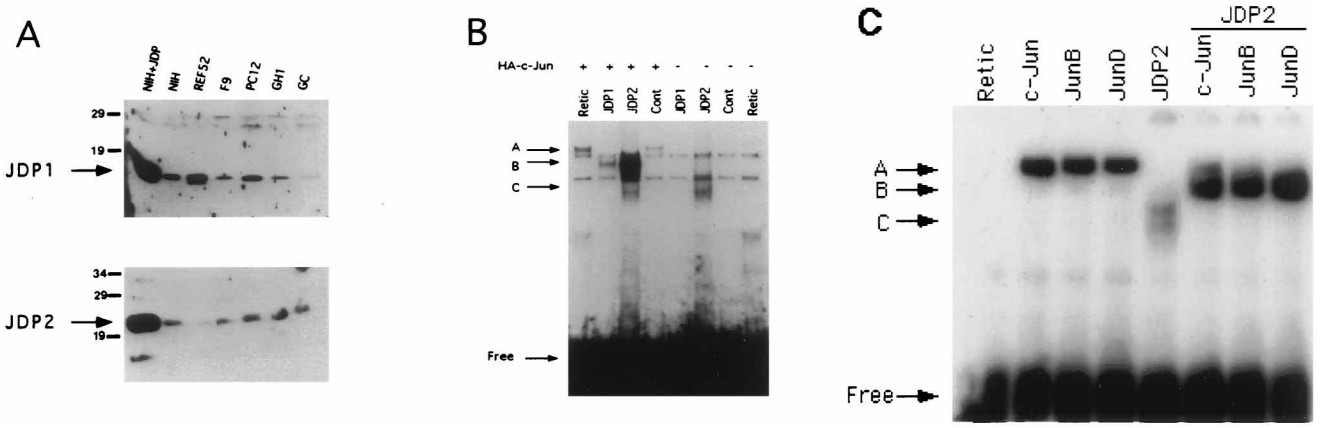

FIG. 5. JDP1 and JDP2 are ubiquitously expressed Jun dimerization partners. (A) Western blot analysis of different cell lysates with JDP1 and JDP2 antisera. Nuclear extracts of various mouse (NIH 3T3 and F9) and rat (REF52, PC12, GH1, and GC) cells were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted with JDP1 and JDP2 antibodies. Extracts of NIH 3T3 cells transfected with either JDP1 or JDP2 expression plasmids were also included. The endogenous JDP1 and JDP2 proteins migrate at a position identical to that of those encoded by the transfected plasmids. (B) JDP1 and JDP2 DNA binding activities and dimerization with c-Jun. An electrophoretic mobility shift assay was performed with cell-free translated c-Jun and JDP1 and JDP2. Clone 2, used as a control (Cont), encodes Ha-Ras and shows no interaction with c-Jun. Retic, unprogrammed reticulocyte lysate. The migration positions of the Jun-Jun homodimer (A), Jun-JDP1 and Jun-JDP2 heterodimers (B) and JDP2-JDP2 homodimers (C) bound to the *jun*-TRE oligonucleotide probe are indicated. (C) Interaction of JDP2 with JunB and JunD. The experimental procedures are as in panel B. A, B, and C indicate the migration positions of Jun-Jun, JDP2-JDP2, and Jun-JDP2, respectively.

relative of JDP2, LRF1/ATF3, is encoded by an immediate-early gene (19), we examined the effect of serum stimulation on JDP2 expression. Unlike c-Fos, whose expression was potently induced by serum in serum-deprived NIH 3T3 or PC12 cells, the expression of JDP2 was constitutive (Fig. 7B and data not shown). The amount of c-Jun associated with JDP2 was slightly decreased in response to serum stimulation, while the amount of c-Jun associated with c-Fos was strongly elevated by serum stimulation (Fig. 7C). As serum addition also increased the total amount of c-Jun (data not shown), these results indicate that after serum stimulation, most of the c-Jun is associated with c-Fos or similar Fos proteins, while in serum-deprived cells, c-Jun–JDP2 complexes are prevalent. Interactions between these proteins were also examined by a *jun*-TRE pulldown assay (Fig. 7D). The amount of JDP2 brought down by *jun*-TRE beads from serum-deprived cell extracts is 30% higher than the amount precipitated from extracts of serum-stimulated cells (Fig. 7D). Comparable amounts of c-Jun were brought down from extracts of either nonstimulated or serum-stimulated cells (Fig. 7D). c-Fos, however, was precipitated by the *jun*-TRE beads only from extracts of serum-stimulated cells (Fig. 7D). Since Jun-Fos heterodimers are more stable than Jun-Jun homodimers and, as shown in Fig. 5B, Jun-JDP2 heterodimers also seem to be more stable than Jun-Jun homodimers, as reflected by high levels of DNA binding activity, these results further support the notion that a major compo-

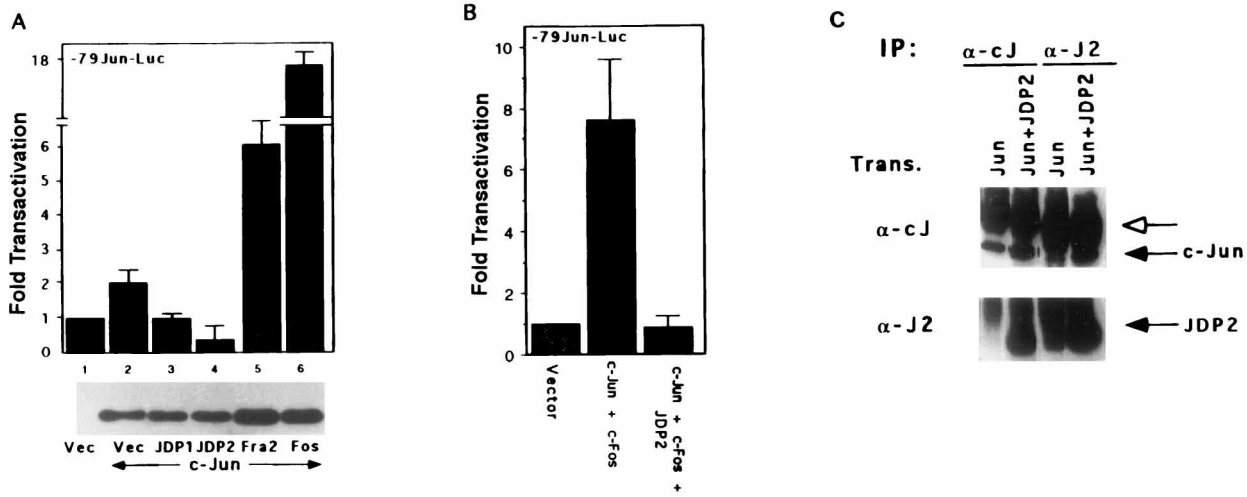

FIG. 6. JDP2 is an AP-1 repressor subunit. (A) NIH 3T3 cells were cotransfected with *jun*-Luc reporter (1 μg) together with either an empty vector or expression vectors encoding HA-tagged c-Jun (1 μg), and JDP1, JDP2, Fra2, or c-Fos (2 μg each) as indicated. At 24 h after transfection, the cells were collected and luciferase activities were determined. The results represent the means of three independent experiments. Immunoblotting of the respective transfected cell extracts with anti-HA antibodies (bottom panel) indicates that none of the JDP and Fos proteins affects c-Jun levels. (B) NIH 3T3 cells were cotransfected with *jun*-Luc and either empty expression vector or expression vectors for c-Jun, c-Fos, or JDP2 as indicated. The results represent the means of three independent experiments. (C) JDP2 and c-Jun associate in transfected cells. Nuclear extracts of NIH 3T3 cells transfected with the indicated expression vectors for c-Jun and JDP2 were precipitated with either c-Jun or JDP2 antibodies as indicated. After 24 h, the cells were collected and luciferase activity was determined. The immunocomplexes were collected, separated by SDS-PAGE, and immunoblotted with either c-Jun and JDP2 antibodies. The migration positions of c-Jun and JDP2, as well as molecular weight markers, are indicated.

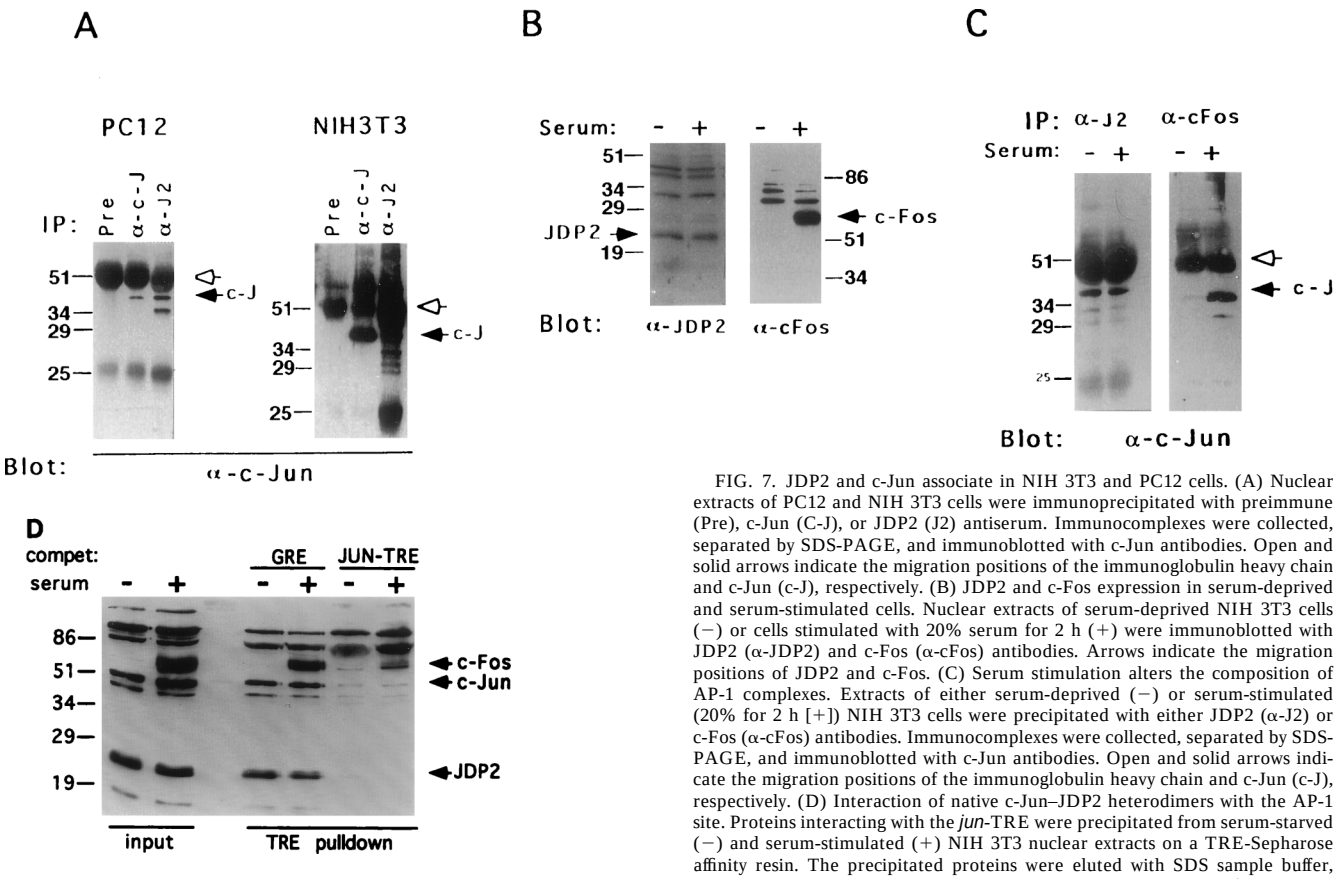

FIG. 7. JDP2 and c-Jun associate in NIH 3T3 and PC12 cells. (A) Nuclear extracts of PC12 and NIH 3T3 cells were immunoprecipitated with preimmune (Pre), c-Jun (C-J), or JDP2 (J2) antiserum. Immunocomplexes were collected, separated by SDS-PAGE, and immunoblotted with c-Jun antibodies. Open and solid arrows indicate the migration positions of the immunoglobulin heavy chain and c-Jun (c-J), respectively. (B) JDP2 and c-Fos expression in serum-deprived and serum-stimulated cells. Nuclear extracts of serum-deprived NIH 3T3 cells (−) or cells stimulated with 20% serum for 2 h (+) were immunoblotted with JDP2 (α-JDP2) and c-Fos (α-cFos) antibodies. Arrows indicate the migration positions of JDP2 and c-Fos. (C) Serum stimulation alters the composition of AP-1 complexes. Extracts of either serum-deprived (−) or serum-stimulated (20% for 2 h [+]) NIH 3T3 cells were precipitated with either JDP2 (α-J2) or c-Fos (α-cFos) antibodies. Immunocomplexes were collected, separated by SDS-PAGE, and immunoblotted with c-Jun antibodies. Open and solid arrows indicate the migration positions of the immunoglobulin heavy chain and c-Jun (c-J), respectively. (D) Interaction of native c-Jun–JDP2 heterodimers with the AP-1 site. Proteins interacting with the *jun*-TRE were precipitated from serum-starved (−) and serum-stimulated (+) NIH 3T3 nuclear extracts on a TRE-Sepharose affinity resin. The precipitated proteins were eluted with SDS sample buffer, separated by SDS-PAGE, and immunoblotted with a mixture of anti-c-Fos, anti-c-Jun and anti-JDP2 antibodies. Excess glucocorticoid response element (GRE) and *jun*-TRE oligonucleotides were used as nonspecific and specific competitors, respectively. The migration positions of c-Fos, c-Jun, and JDP2 are indicated.

nent of AP-1 activity associated with *jun*-TRE in nonstimulated cells is the c-Jun–JDP2 heterodimer. In serum-stimulated cells, c-Jun–c-Fos heterodimers are more abundant mostly due to Fos induction. Because in both coimmunoprecipitation and *jun*-TRE pulldown experiments the amounts of JDP2 that was brought down decrease following serum stimulation, whereas c-Fos is induced, it is reasonable to suggest that c-Fos has displaced JDP2 from the Jun-JDP2 complex to form Jun–c-Fos heterodimers.

DISCUSSION

Our results demonstrate the utility of the SRS in identifying novel dimerization partners and in studying protein-protein interactions. While most such studies can be performed with the existing two-hybrid system (1, 16), the SRS may be the method of choice in studying interactions that involve transcriptional activators or repressors, because it is not based on a transcriptional readout. In addition, certain proteins may function more physiologically when expressed in the cytoplasm rather than the nuclear milieu. For example, the SRS should be more suitable for examining interactions between proteins that require modification by cytoplasmic or membrane-associated enzymes. Moreover, several proteins were toxic to yeast in the conventional two-hybrid system (1). Toxicity may disappear when these proteins are fused to hSos and expressed in the cytoplasm (e.g., GHF1 [18a]).

We have used the SRS to identify two novel regulators of c-Jun function, JDP1 and JDP2. JDP2 represents the first c-Jun repressor protein isolated so far. Our findings indicate that JDP2 has properties consistent with a role in maintaining low basal levels of AP-1 transcriptional activity in nonstimulated cells. First, JDP2 is present as a heterodimer with c-Jun. Second, it represses c-Jun transcriptional activity by competing with activators for c-Jun binding. JDP2 probably is likely to harbor an active repression domain rather than merely lacking an AD, because its effect on c-Jun transcriptional activity is different from that of Fra2. Although Fra2 lacks an AD, it still enhances c-Jun-mediated activation through its effect on dimer stability, albeit to a lesser extent than c-Fos, which contains an AD (13, 42). Third, it enhances the binding of c-Jun to AP-1 sites (both to *col*-TRE and to *jun*-TRE), thereby providing further inhibition through competition with transcriptionally active heterodimers for TRE binding. While JDP2 is expressed constitutively, serum stimulation results in a higher proportion of c-Jun–Fos heterodimers in comparison to c-Jun–JDP2 dimers. This effect is probably due to de novo synthesis of both c-Jun and c-Fos. The replacement of repressive c-Jun–JDP2 dimers by activating c-Jun–c-Fos dimers would result in a much higher amplitude of AP-1 activity than would be produced by merely increasing c-Jun and c-Fos synthesis. The negative regulation of AP-1 activity by JDP2 resembles the regulation of Myc-mediated transcription. In that case, serum stimulation and c-Myc induction replace repressive Mad-Max dimers by activating Myc-Max dimers (7, 8). We propose that JDP2 acts in a similar way to Mad to inhibit c-Jun activity. In this light, JDP2 could be used as a general inhibitor involved in cell cycle

arrest and terminal differentiation and, like Mad, may have tumor suppressor activity.

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