Modulation of JunD-AP-1 DNA Binding Activity by AP-1-associated Factor 1 (AF-1)*

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AP-1-associated factor 1 (AF-1), is a novel protein complex that dramatically enhances the assembly of JunD-containing dimers onto AP-1 consensus sites. We describe the partial purification of AF-1 from nuclear extracts of the T-cell line MLA 144 by ionic, hydrophobic and gel filtration chromatography. AF-1 is a DNA-binding protein composed of low molecular mass polypeptides of 7–17 kDa that exists in solution as a 34-kDa complex. JunD interactions with DNA are accelerated in the presence of AF-1 through the formation of a true tri-molecular complex with JunD dimers and DNA that assembles much more rapidly on DNA than JunD alone. DNA binding analysis of AF-1 interaction with JunD-AP-1 and DNA shows that AF-1 increases the DNA binding affinity of JunD for AP-1 sites over 100-fold. DNA cleavage footprint analysis of isolated AF-1-JunD DNA complexes shows that the ternary complex makes nearly twice as many contacts with DNA than JunD dimers alone. AF-1 interacts readily, but differentially with Jun homodimers and Jun-Fos heterodimers. These findings distinguish AF-1 as a significant protein-specific modulator of AP-1-JunD in T-cells.

A central goal in the field of signal transduction biology is to elucidate the mechanisms through which short-lived signals, emanating from the surface of the cell, can be conveyed internally to effect long term changes in cellular behavior. Most of these changes are brought about by modulation of gene expression at the level of transcription (1). The AP-1 transcription factors are a ubiquitous class of gene regulatory factors that have a leading role in controlling widely diverse cellular processes (2, 3). This class of basic leucine zipper (bZip)1 DNA-binding proteins binds specifically to sequences related to the pseudopalindromic AP-1 consensus site (5'-TGA C/G TCA-3').

In previous work, we isolated a cellular fraction from 0.8 M NaCl T-cell nuclear extracts that contained a novel activity that dramatically stimulated JunD-AP-1 DNA binding activity (29). Within this fraction, significant stimulatory activity was found to reside in a set of 29–23-kDa polypeptides. In this current work we describe the identification, partial purification, and properties of a second active component of this 0.8 M NaCl fraction as a multimeric DNA-binding protein composed of 7–17-kDa polypeptides. This factor, termed AP-1-associated factor 1 (AF-1), enhances JunD-AP-1 DNA binding by forming a tri-molecular complex with AP-1 dimers and DNA that binds with a 100-fold higher affinity than JunD alone. Interestingly, inducible AF-1-Jun complexes can be demonstrated in nuclear extracts from mitogen-stimulated T-cells that are partially inhibited in the presence of cyclosporine A.

EXPERIMENTAL PROCEDURES

Materials—Diisopropyl fluorophosphate, leupeptin, pepstatin A, PMSF, Hepes, Tween 20, BSA, NaCl, and poly[dICl] were purchased from Sigma. DEAE-53 cellulose was from Whatman. Protein A-agarose, Triton X-100, and Bio-Rex 70 resin were from Bio-Rad. Phenyl-Sepha-rose CL-4B was from Pharmacia Biotech Inc. Affinity-purified antibodies against JunD were prepared as described (29).

Cells and Media—The Jurkat human T-cell line and MLA144 cells (gibbon T-cell lymphoma) were grown in RPMI 1640 medium containing 10% fetal calf serum. Cells were harvested by centrifugation at 1000 x g for 10 min, and the pellets were washed three times with 3 volumes of cold PBS prior to preparation of nuclear extracts.

Preparation of Nuclear Extracts—Nuclear extracts were prepared from resting or mitogen-stimulated Jurkat T-cells by an extensive modification of the procedure described by Ohlsson and Edlund (30). Cells (approximately 1–3 x 10^9) were harvested and washed as described above. The cells were then resuspended in 5 volumes of Buffer A (10 mM KCl, 1.5 mM MgCl₂, 4 mM BME, 0.5 mM PMSF, 10 µg/ml leupeptin, 10 mM Hepes, pH 7.5) and transferred to a 1.5-ml Eppendorf tube and allowed to stand for 10 min at 4 °C. The cells were then collected by centrifugation at 1000 x g for 10 min. at 4 °C. The supernatant was...
removed, and the cells were resuspended in 2 volumes of Buffer A and homogenized with 16 strokes of a 1.8-mm (fitted Teflon pestle) driven at 1500 rpm by a hand-held 0.75-watt power drill. Nuclei were collected by centrifugation at 100,000 × g for 10 min at 4 °C, and the supernatant was removed. The isolated nuclei were resuspended in 5 volumes of Buffer B (0.2 mM EDTA, 4 mM BME, 0.5 mM PMSF, 0.05% Tween, 0.25 mM EDTA, 0.2 M NaCl, 10 mM Hepes, pH 7.5). The suspension was then allowed to incubate at 4 °C for 30 min. The nuclear suspension was then clarified by centrifugation at 100,000 × g for 60 min at 4 °C. The resulting supernatant was quickly recovered prior to re-swelling of the nuclear pellet and assayed directly for DNA binding activity.

Preparation of Anti-JunD Antibody Immunopurified Cells—Cells that had been previously harvested at 70% confluence were resuspended in 2 volumes of 100% ethanol, 1 volume of 20% glycerol, 0.5 mM PMSF, 0.5 mM PMSF, 0.5 mM PMSF, and 2 volumes of 20 mM Hepes, pH 7.5. The suspension was then allowed to incubate at 4 °C for 30 min. The mixture was then centrifuged at 300,000 × g for 1 h. The antibody-coated agarose beads were washed twice with buffers containing 100 mM NaCl, 0.2% Triton X-100, and 0.5 mM sodium azide. A second nonspecific (anti-glutathione S-transferase antibody) antibody column was prepared from 10 ml of protein A-agarose and 30 mg of anti-GST antibodies.

Immunopurification of JunD from T-cells—Nuclear extracts of MLA 144 T-cells were prepared as described previously (29), and diazylated against PBS, 0.2% Triton X-100, 0.5 mM PMSF, and 1 mM azide, pH 7.5, for 3 h at 4 °C. The extract was then passed over a 10-ml anti-GST column, and the resulting flow-through was then passed over a 2-ml (5 mg/ml) anti-JunD affinity column. After washing successively with 25 column volumes of 0.5 mM NaCl, 0.1 M glycine, 1 mM sodium azide, 200 ml of buffer (containing 0.5 mM NaCl, 1 mM sodium azide, 0.5 mM NaCl, 10 mM Hepes, pH 7.5), and finally equilibrated with 5–6 column volumes of buffer containing PBS, 0.2% Triton X-100, and 1 mM sodium azide. A second nonspecific (anti-glutathione S-transferase antibody) antibody column was prepared from 10 ml of protein A-agarose and 30 mg of anti-GST antibodies.

Immunopurification of JunD from T-cells—Nuclear extracts of MLA 144 T-cells were prepared as described previously (29), and diazylated against PBS, 0.2% Triton X-100, 0.5 mM PMSF, and 1 mM azide, pH 7.5, for 3 h at 4 °C. The extract was then passed over a 10-ml anti-GST column, and the resulting flow-through was then passed over a 2-ml (5 mg/ml) anti-JunD affinity column. After washing successively with 25 column volumes of 0.5 mM NaCl, 0.1 M glycine, 1 mM sodium azide, 200 ml of buffer (containing 0.5 mM NaCl, 1 mM sodium azide, 0.5 mM NaCl, 10 mM Hepes, pH 7.5), and finally equilibrated with 5–6 column volumes of buffer containing PBS, 0.2% Triton X-100, and 1 mM sodium azide. A second nonspecific (anti-glutathione S-transferase antibody) antibody column was prepared from 10 ml of protein A-agarose and 30 mg of anti-GST antibodies.

RESULTS
Immunopurification of JunD—Prior studies had identified an activity or activities, isoalted from 0.8 M NaCl extracts of MLA144 T-cell nuclei, that dramatically enhanced the DNA binding activity of JunD (AP-1) (29). Similar non-Fos, non-Jun activity could also be found as contaminating components in DNA affinity-purified preparations of JunD (29, 33). The identification of these activities was based solely on the ability of the isolated fragments to stimulate specific binding of purified JunD to oligonucleotides containing the GALV-TRE sequence. To facilitate a more detailed study of this stimulatory activity in the current studies, it was therefore important to utilize preparations of purified JunD that were free of any contaminating components that could interfere with the assessment of regulated Jun-DNA binding activity. This required obtaining active preparations of purified JunD via means that were independent of DNA binding. Such requirements necessarily excluded DNA affinity-purified preparations of T-cell JunD as a suitable substrate for study. Although an attractive alternative, preparations of recombinant JunD contain very low percentages of active molecules. Due to their low DNA binding activity, these preparations must be used at very high protein concentrations. The possibility that the over-whelmig excess of inactive molecules could interfere with proper assessment of the regulated function of the active population of JunD rendered recombinant sources of JunD even less suitable than the DNA affinity-purified JunD. JunD iso-
lated from T-cell nuclear extracts by immunoaffinity purification was found to be the most useful and revealing substrate for the task of assessing the modulation of AP-1 DNA binding activity by various purified cellular components.

Cellular T-cell JunD was obtained at high purity from nuclear extracts of gibbon ape T-cells by immunoaffinity purification (see “Experimental Procedures”). Nuclear extracts rich in JunD DNA binding activity were generated from isolated gibbon ape T-cell MLA144 nuclei (29). After first processing over a nonspecific anti-GST antibody column, the extracts were passed over an affinity column that had been previously coupled to purified polyclonal anti-JunD antibodies at high concentration. Subsequently the column was washed with high salt buffers and eluted with 4 M MgCl₂ (see “Experimental Procedures”). JunD purified in this manner was obtained in high purity and demonstrated the same predominant 43-kDa and 38-kDa polypeptides previously described in DNA affinity-purified JunD (29, 33). Moreover, this purified T-cell JunD was highly active, specific, and bound DNA at low protein concentrations. This immunoaffinity-purified form of JunD therefore was used throughout the purification and characterization of AP-1.

**Purification and Properties of AF-1**—AF-1 was purified from isolated T-cell nuclei by differential salt extraction followed by batchwise anion exchange chromatography, phenyl-Sepharose hydrophobic chromatography, gradient and stepwise cation exchange chromatography, ending with gel filtration chromatography (see “Experimental Procedures”). Isolated fractions were assayed for the ability to stimulate JunD DNA binding activity by EMSA. The peak of AF-1 activity in the final gel filtration step eluted with a molecular mass of approximately 34 kDa (see “Experimental Procedures”). SDS-PAGE analysis of peak fractions demonstrated a class of low molecular mass polypeptides ranging from approximately 7 to 17 kDa that consistently co-migrated with AF-1 activity (ternary complex formation with JunD and DNA; see Fig. 2A) at each step (Fig. 1, left). The apparent discrepancy in the native molecular mass of AF-1 and the size of the AF-1 polypeptides by SDS-PAGE suggest that AF-1 is multimeric, a highly elongated molecule, or both.

**EMSA analysis of purified AF-1**—AF-1 in the absence of JunD reveals an intrinsic DNA binding activity that is independent of added factors and can be detected as a rapidly migrating DNA-protein complex (Fig. 1, right).

**AF-1 Forms a Stable Ternary Complex with JunD Dimers and DNA**—The addition of increasing amounts of AF-1 to fixed amounts of JunD homodimers generates increased JunD DNA binding activity associated with the gradual formation of a ternary AF-1-JunD-DNA complex with significantly decreased DNA recognition activity. The apparent discrepancy in the native molecular mass of AF-1 and the size of the AF-1 polypeptides by SDS-PAGE suggests that AF-1 is multimeric, a highly elongated molecule, or both.

**FIG. 1. Purification and properties of AF-1.** A, left panel, SDS-PAGE analysis of purified AF-1. Arrows indicate position of polypeptides that consistently co-migrated with AF-1 activity. Asterisk indicates position of a contaminating polypeptide whose peak elutes with a smaller size (Rₚ) than the AF-1 polypeptides (see “Experimental Procedures”). Right panel, EMSA analysis of purified AF-1 interaction with GALV-TRE DNA on a 4% acrylamide gel. Arrows indicate free DNA and DNA-protein complexes.

**FIG. 2. AF-1 polypeptide(s) form ternary complexes with JunD dimers and DNA.** A, 8% acrylamide EMSA analysis of the addition of increasing amounts (2.5–20 ng) of AF-1 to a fixed amount (0.25 ng) of immunopurified JunD. Arrows show the positions of JunD binary complexes with DNA and AF-1-JunD ternary complexes as indicated. Electrophoresis was carried for 16 h to provide maximum resolution of JunD and AF-1-JunD complexes, as a result, free DNA and AF-1-JunD complexes were run off the gel and are, therefore, not present. B, competition analysis and comparison of DNA specificity of JunD and AF-1-JunD complexes for DNA. Purified JunD (0.25 ng) was incubated with 0.2 ng of ³²P-labeled GALV-TRE in the presence or absence of 20 ng of AF-1 (as indicated) and 50 ng of the either wild type (59 + 60) or GALV-TRE sequences that were mutated by sets of 4 base transversion along the length of the sequence (see upper panel). The boxed area (upper panel) outlines the 7-base pair TRE consensus sequence. C, AF-1-dependent complexes with JunD contain AF-1 polypeptides. JunD (0.2 ng) was incubated with 8 ng of ³²P-labeled GALV-TRE in the presence of absence of 20 ng of AF-1 as indicated. JunD-DNA and AF-1-JunD-DNA complexes were isolated by EMSA (lower panel) and cross-linked in situ by UV irradiation (see “Experimental Procedures”). DNA-protein adducts were excised, eluted, and analyzed by SDS-PAGE on a 15–20% gradient gel (upper panel). Protein-DNA adducts migrating at approximately 53 and 29 kDa in both lanes (arrowheads) represent DNA cross-linked JunD and JunD degradation products observed previously (29). Arrow indicates the position of the 19-kDa protein-DNA cross-linked adduct that is present exclusively in the AF-1-JunD-DNA complex.
AF-1 Preferentially Forms Ternary Complexes with JunD and Increases the Rate of JunD Assembly onto DNA—When increasing amounts of JunD are added to a fixed concentration of AF-1 and DNA, a dramatic increase in DNA binding activity occurs with a concomitant decrease in the amount of binary AF-1-DNA complexes (Fig. 4A). The AF-1-JunD-DNA complexes show significantly higher binding than comparable amounts of JunD alone and the gradual decrease in the amount of binary AF-1-DNA complexes indicates that AF-1 has a much higher affinity and/or specificity for DNA complexes with JunD than for DNA alone.

Semi-quantitative analysis of the rate of assembly of binary JunD-DNA versus ternary AF-1-JunD-DNA complexes shows that AF-1-JunD complexes assemble onto DNA much more rapidly than JunD alone (Fig. 4B). Time-constrained binding of JunD to a TRE in the presence or absence of AF-1 shows that, while the binding of JunD to the TRE is maximum at 5 min, the formation of the AF-1-JunD complexes occurs much faster (less than 1 min). In fact, the rate of assembly of the AF-1-JunD complex is so rapid, it cannot be measured within the limitation of the assay. Interestingly, the dissociation rates of both the JunD and the AF-1-JunD complexes appear to be very similar (data not shown). Thus, AF-1 exerts its effects on JunD DNA binding by increasing the contacts it makes with DNA and these AF-1-dependent contacts promote a more rapid assembly of AP-1 onto DNA, while having little effect on the half-life of the complex.

AF-1 Shows Differential Binding Activity for Jun Homodimers and Jun-Fos Heterodimers—The extent of AF-1’s ability to enhance AP-1 DNA binding activity is dependent on the context of the AP-1 members within the dimeric complex. When measured by EMSA at low concentrations, AF-1 readily stimulates the binding of both purified T-cell JunD and recombinant GST-JunD but has little effect on recombinant c-Jun (Fig. 5A). Similarly, while low concentrations of AF-1 stimulate the binding activity of both purified Jurkat T-cell JunD homodimers and Jun-D-Fos heterodimers, it shows little effect on the DNA binding activity of either recombinant c-Jun homodimer or c-Jun heterodimerized with c-Fos (Fig. 5B). Although these differences are much less pronounced at higher concentrations of AF-1 (data not shown), they indicate two important points about the mechanism AF-1 interaction. (i) AF-1 can associate with both Jun-containing homodimers and heterodimers containing Fos, and (ii) AF-1 interaction with AP-1 shows protein specificity and will differ depending on the context of the monomeric components present in the dimeric complex.

Inducible AF-1-JunD Complexes Are Present in Nuclear Extracts from PHA/PMA-stimulated Jurkat T-cells—EMSA analysis of crude nuclear extracts prepared from PHA/PMA-stimulated Jurkat T-cells reveals the presence of an inducible AP-1/TRE complex in addition to a slower migrating AP-1-containing complex that comigrates precisely with the AF-1-JunD complex reconstituted from purified JunD and AF-1 components (Fig. 6, A and B). Notably, this complex is highly induced above the levels detectable in unstimulated Jurkat T-cells. Moreover, the inducible AF-1-Jun complex is partially (approximately 33%) inhibited by prior treatment with 100 nM cyclosporine (Fig. 6A). Antibody supershift analysis of these complexes with anti-JunD and anti-Fos antibodies indicates that the predominant complex in uninduced T-cells is composed mainly of Jun homodimers, while the more strongly
inducible and slower migrating AF-1/AP-1 complex contain both Jun and Fos (Fig. 6B). These data provide strong evidence that the AF-1-JunD complexes reconstituted from purified AF-1 and cellular JunD are representative of the in vivo complexes found in nuclear extracts from PHA/PMA-induced Jurkat T-cells.

**DISCUSSION**

This report describes the identification, partial purification, and characterization of a novel protein, termed AF-1, that dramatically facilitates the assembly of AP-1 dimers onto DNA. At present, the actual stoichiometry of the AF-1 subunits in the AF-1/AP-1 complex remains undetermined until sufficient amounts of either purified or recombinant protein become available for quantitative study. Preliminary evaluation of AF-1 DNA binding activity in the absence of JunD or other AP-1 complexes indicates that AF-1 forms complexes with DNA that have little specificity in the absence of other factors (data not shown). The avidity that AF-1 shows for JunD-DNA complexes over DNA alone (see Fig. 4A) is consistent with this observation.

The ability of AF-1 to increase AP-1 DNA binding by primarily influencing the on rate of AF-1-JunD DNA binding, while having little or no effect on the half-life of the complex, could occur by two basic mechanisms. By the first model, AF-1 forms a binary complex with JunD dimers prior to binding to DNA. This binary complex could then assemble onto DNA in a more thermodynamically favorable fashion that could occur through stabilization of AP-1 dimer formation, relative lowering of the activation energy barrier for binding to DNA, or both. An essential component of this model is that AF-1 is capable of forming stable complexes with AP-1 dimers that can alter the dimer monomer equilibrium in the absence of DNA. Thus far we have not been able to detect any evidence of protein-protein interactions between AF-1 and either immunopurified or recombinant JunD. By a second model, AF-1 has insignificant affinity for JunD dimers alone but binds avidly to binary complexes.
JunD-DNA complexes. Accordingly, JunD dimers would also be expected to bind with similar avidity to AF-1-DNA complexes. By this model either AF-1 or JunD binds preferentially to the extended DNA-protein surface created by its bound congener and DNA. This mode of interaction is very similar to that proposed for the tight interaction between calcineurin and the composite drug-protein surface created by the formation of cyclosporine-immunophilin complexes (34). These models are very similar to those proposed for the mechanism of action of TAX-1 (24).

The observation that AF-1 acts differentially with AP-1 dimers depending on the context of the individual AP-1 dimerization partners is quite intriguing. At the very least, it indicates that AF-1 interactions with dimeric AP-1 shows protein specificity. Whether or not the differential preference of AF-1 for dimers containing JunD, in comparison to those containing c-Jun, reflects a true “Jun family member preference” or is more a reflection of the differential stability of recombinant preparations of c-Jun protein remains to be investigated. It should be noted that these differences appear to be less striking when AF-1 is added to dimers of JunD or c-Jun at higher concentrations. The biological relevance of these differences will be better addressed when recombinant preparations of AF-1 become available for eucaryotic expression.

EMSA DNA binding analysis by extended electrophoresis on 8% polyacrylamide gels has added a new level of resolution to the analysis of AP-1 protein-DNA complexes. Under conventional conditions (4% acrylamide electrophoresis at 24 °C for 1 h), the protein-DNA complexes now known to have altered mobility and composition migrate as superimposed or dissociated complexes during electrophoresis. As a result, the AF-1-Jun-DNA ternary complexes appear indistinguishable from the binary Jun-DNA complexes. In addition to providing greater resolution, the electrophoretic separation of the protein-DNA complexes on 8% acrylamide at 4 °C provides a greater stability to the relative steady state ratio of binary and ternary complexes formed at equilibrium prior to electrophoresis. Nonetheless, at subsaturating concentrations of AF-1, this ratio appears to decay as the complexes fall apart during electrophoresis. The net result is an apparent increase in the relative amount of binary complexes at the completion of the electrophoresis. Such transitions are readily apparent in the EMSA analyses shown in Figs. 2A, 5, and 6A.

EMSA separation of crude nuclear extracts derived from Jurkat T-cells on 8% acrylamide reveals the presence of two distinct inducible TRE-binding complexes. Both complexes contain Jun. One co-migrates with purified AP-1 dimers and DNA, and the second complex migrates with a mobility that is identical to the AF-1-JunD complexes reconstituted from separately purified components (Fig. 6, A and B). The presence of AF-1 within T-cell activation inducible AP-1 complexes reveals a substantial role for AF-1 as modulator of AP-1 function in T-cells. The partial inhibition of inducible AF-1-Jun complexes by cyclosporine A treatment is quite intriguing and suggests that some step in the pathway to the assembly of the ternary AF-1-Jun complex may be modulated by cyclosporine A.

AF-1 represents one of two separate activities in the 0.8 M NaI extract of isolated T-cell nuclei that augment AP-1 DNA binding activity. The previously identified 29–23-kDa polypeptides found to augment JunD binding activity (see Ref. 29) show no evidence of ternary complex formation and do not alter the mobility of Jun complexes by extended EMSA electrophoresis on 8% acrylamide gels.3 By contrast, AF-1 shifts the mobility of Jun complexes and the AF-1-dependent complexes show increased contacts with sequences flanking the TRE consensus site. Currently, the mechanism of action of the 29–23-kDa polypeptides remains undefined.

It is not clear whether or not AF-1 will act as a negative transcriptional regulator or function positively. The observation that AF-1-Jun-like complexes are strongly induced during T-cell activation argues for a more prominent role as a positive

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[3] C. Powers and K. Gardner, unpublished observation.
regulator of AP-1 function. Like TAX-1, AF-1 could effect the DNA binding properties of multiple basic leucine zipper family members. The predominant role of AF-1 as an accessory protein that accelerates the rate of assembly of transcription factor complexes onto DNA could have profound influences on gene expression pathways that must generate a rapid response once threshold levels of active downstream signal transduction targets have been achieved. AF-1 could have a role in facilitating transcription factor entry and transcriptional co-factor function within the complicated and dynamic chromatin structure that surrounds rapidly inducible genes. Further purification, cloning, and functional characterization of AF-1 will provide critical insights into the mechanisms governing these important cellular processes.

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