A Dominant Negative to Activation Protein-1 (AP1) That Abolishes DNA Binding and Inhibits Oncogenesis*

(Received for publication, November 14, 1996, and in revised form, April 11, 1997)

Michelle Olive, Dmitry Krylov, Deborah R. Echlin‡§, Kevin Gardner‡, Elizabeth Taparowsky‡, and Charles Vinson

From the Laboratory of Biochemistry and §Laboratory of Pathology, NCI, National Institutes of Health, Bethesda, Maryland 20892 and the ¶Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

We describe a dominant negative (DN) to activation protein-1 (AP1) that inhibits DNA binding in an equimolar competition. AP1 is a heterodimer of the oncogenes Fos and Jun, members of the bZIP family of transcription factors. The DN, termed A-Fos, consists of a newly designed acidic amphipathic protein sequence appended onto the N-terminus of the Fos leucine zipper, replacing the normal basic region critical for DNA binding. The acidic extension and the Jun basic region form a heterodimeric coiled coil structure that stabilizes the complex over 3000-fold and prevents the basic region of Jun from binding to DNA. Gel shift assays indicate that A-Fos can inactivate the DNA binding of a Fos:Jun heterodimer in an equimolar competition. Transient transfection assays indicate that A-Fos inhibits Jun-dependent transactivation. Both the acidic extension and the Fos leucine zipper are critical for this inhibition. Expression of A-Fos in mouse fibroblasts inhibits focus formation more than colony formation, reflecting the ability of A-Fos to interfere with the AP1 biological functions in mammalian cells. This reagent is more potent than a deletion of either the Fos or Jun transactivation domains, which has been used previously as a dominant negative to AP1 activity.

The activation protein-1 (AP1) transcription factors are immediate early response genes involved in a diverse set of transcriptional regulatory processes (1). The AP1 complex consists of a heterodimer of a Fos family member and a Jun family member. This complex binds the consensus DNA sequence (TGAGTCA) (termed AP1) sites found in a variety of promoters (2, 3). The Fos family contains four proteins (c-Fos, Fos-B, Fra-1, and Fra-2) (4–6), while the Jun family is composed of three (c-Jun, Jun-B, and Jun-D) (7–10). Fos and Jun are members of the bZIP family of sequence-specific dimeric DNA-binding proteins (11). The C-terminal half of the bZIP domain is amphipathic, containing a heptad repeat of leucines that is critical for the dimerization of bZIP proteins (12, 13). The N-terminal half of the long bipartite α-helix is the basic region that is critical for sequence-specific DNA binding (14–16).

To dissect the function of the AP1 complex in cellular processes, investigators have used dominant negatives (DNs) to AP1 consisting of a deletion of the transactivation domain of either a Jun family member (17–19) or a Fos family member (20, 21). These truncated Fos or Jun proteins dimerize with endogenous transcription factors, which results in the loss of AP1 activity (21, 22). A conceptual disadvantage with this strategy is that the heterodimer between the endogenous transcription factor and the dominant negative still binds DNA, which makes it difficult to document a change in DNA occupancy that correlates with the expression of the dominant negative. A dominant negative that is deleted for the DNA binding domain would overcome this type of problem, but such potential dominant negatives do not work well because of the stabilization that occurs when bZIP proteins bind DNA (16, 23).

We are interested in developing DNAs (24) that stoichiometrically inhibit the sequence-specific DNA binding of the AP1 complex. These reagents should inhibit AP1 DNA binding in vivo, thus allowing us to monitor occupancy of AP1 cis elements in vivo (25).

We previously demonstrated that the DNA binding of the bZIP protein C/EBP could be inhibited stoichiometrically by appending an amphipathic acidic extension to the N-terminus of the C/EBP leucine zipper (26). We explored the generality of this strategy by appending the same acidic extension onto the Fos leucine zipper. This construct (4H-Fos) was not able to inhibit AP1 DNA binding in an equimolar competition. This paper describes a newly designed amphipathic acidic extension (termed A- or N4H-), which, when appended onto the N-terminus of the Fos leucine zipper, is able to inhibit the DNA binding of AP1 in an equimolar competition. When expressed in mammalian cells, A-Fos inhibits Jun-dependent transactivation and dramatically reduces Ha-ras-mediated cellular transformation in a leucine zipper-dependent fashion.

EXPERIMENTAL PROCEDURES

Proteins—The Fos, Jun, VBP, and CREB bZIP domains were constructed by polymerase chain reaction and cloned into the prokaryotic expression vector pT5 as NdeI-HindIII fragments (26). All of the proteins have a 13-amino acid N-terminal c/10 leader ASM-TGGQQGMGRDP.

The human Fos bZIP domain spans from Lysα28 to Aspβ28, chicken Jun bZIP domain spans from Serε28 to Pheε125, the natural COOH terminus; chicken VBP bZIP domain spans from Lysδ230 to Leuδ311, the COOH terminus; mouse CREB bZIP domain spans from Leuα74 to Aspε144, the COOH terminus. The C/EBP bZIP domain has been described previously (26).

The protein sequences of the acidic extensions of the dominant negatives are as follows. The last L in the following sequences is the first d position (see Fig. 1) of the Fos leucine zipper (14), and for cloning

This paper is available on line at http://www.jbc.org
HPLC system using a C18 column chromatographed from 0 to 100% as described previously (26), and subsequently purified over a Rainin and those capable of binding DNA were purified over a heparin column. The following VBP, CREB, and C/EBP leucine zippers have been changed to an E to produce a Ndel-HindIII fragments by polymerase chain reaction into the pT5 vector. The construct OH-Fos contains the Fos leucine zipper that spans from Leu165 to Asn203 and also has the mutation Q to E mentioned above.

**Protein Purification—**Proteins were expressed in Escherichia coli, and those capable of binding DNA were purified over a heparin column as described previously (26), and subsequently purified over a Rainin HPLC system using a C18 column chromatographed from 0 to 100% as described previously (26), and subsequently purified over a Rainin HPLC system as just described. The protein purification protocol was modified to purify the Jun bZIP protein. The initial pellet of the Jun sample was resuspended in 1x KCl and centrifuged at 25,000 rpm. The pellet was gently brought to 5 M urea, sonicated, heated at 65 °C for 15 min, and centrifuged, and the supernatant was isolated. The proteins were dialyzed against 20 mM Tris, pH 8.1, 1 mM EDTA and subsequently purified over a heparin column as described previously (26). The molar concentrations were calculated as described previously (26). The AP1 complex was purified from T cells as described previously (45).

**Circular Dichroism—**Tm values were calculated as described previously (46), converted to K社会主义, and G (using a ΔC of -1.4 kcal/mol °C calculated from a Tm versus ΔT plot for all of the proteins used in this study. All thermal melts were reversible. The spectra were recorded in a 0.5-cm cuvette.

**DNA Binding Assay—**Proteins (2 μl of 5 × 10^-6 μl ml) were heated for 10 min at 65 °C in the presence of 1 μM dithiothreitol and added to 20 μl of the gel shift reaction buffer (25 mM Tris (pH 8.0), 50 mM KCl, 0.5 mM EDTA, 2.5 mM dithiothreitol, 1 mM mg/ml bovine serum albumin, 10% glycerol), incubated for 10 min at 25 °C, and then mixed with 8 μg of the probe (46)-labeled double-stranded oligonucleotide containing the AP1 site). The binding complexes were resolved on an 8% polyacrylamide gel in 0.5% TBE buffer at room temperature. The sequence of the probe (32P-labeled double-stranded oligonucleotide containing the AP1 site) is TGACGTCA and the DNA binding sites are in boldface type. The conditions used for the DNA binding assay were identical except that 0.3 μg of salmon sperm DNA. After 2 days, cells were harvested and assayed for chloramphenicol acetyltransferase (CAT) activity. CAT activities were normalized to 1.00. The efficiency of transfection was measured in HepG2 cells. The CAT reporter plasmid p10 used for transfection experiments in Jurkat cells consists of a chimeric c-fos promoter gene fusion carrying the Gibbon ape leukaemia virus-TPA-responsive element enhancer and has been described previously (49). Dominant negative coding sequences (OH-Fos, 4H-Fos, A-Fos, A-VDP, 4H-CREB, A-CREB) were cloned as Ndel-HindIII fragments into the pRC/CMV vector (Invitrogen) modified to contain a N-terminal hemagglutinin epitope (MYPYDVPDYA) pRC/CMV566 or an N-terminal FLAG epitope (MDYKDDDK) and a new polylinker. The Ndel-HindIII fragments were obtained from the proviral expression vector pT5 in which the dominant negatives had been cloned previously (see “Proteins”).

**Indirect Immunofluorescence—**HepG2 cells were cultured in 1-ml slide flasks (Nunc). Cells were fixed in 4% formaldehyde in PBS for 20 min at room temperature followed by methanol for 10 min. After blocking with 3% bovine serum albumin in PBS and 0.1% Tween 20 for 30 min at room temperature, slides were incubated with a 1:200 FLAG M2 antibody and 3% bovine serum albumin in PBS for 2 h, followed by incubation for 1 h with fluorescein isothiocyanate-conjugated rat anti-mouse antibody used at a 1:200 dilution in PBS containing 3% bovine serum albumin. After each incubation with antibodies, cells were extensively washed with PBS, 0.05% Twenn 20 for 10 min each at room temperature.

**Eukaryotic Plasmids—**The eukaryotic expression plasmid containing chicken Jun is driven by the CMV promoter and has been described elsewhere (21). The CAT reporter plasmid containing a single AP1 binding site was constructed by inserting the AP1 consensus site (126) into AP1 (46), converted to K社会主义 values were calculated previously (26) and G (using a ΔC of -1.4 kcal/mol °C calculated from a Tm versus ΔT plot for all of the proteins used in this study. All thermal melts were reversible. The spectra were recorded in a 0.5-cm cuvette.

**DNA Binding Assay—**Proteins (2 μl of 5 × 10^-6 μl ml) were heated for 10 min at 65 °C in the presence of 1 μM dithiothreitol and added to 20 μl of the gel shift reaction buffer (25 mM Tris (pH 8.0), 50 mM KCl, 0.5 mM EDTA, 2.5 mM dithiothreitol, 1 mM mg/ml bovine serum albumin, 10% glycerol), incubated for 10 min at 25 °C, and then mixed with 8 μg of the probe (46)-labeled double-stranded oligonucleotide containing the AP1 site). The DNA binding sites are in boldface type. The conditions used for the DNA binding assay were identical except that 0.3 μg of salmon sperm DNA. After 2 days, cells were harvested and assayed for chloramphenicol acetyltransferase (CAT) activity. CAT activities were normalized to 1.00. The efficiency of transfection was measured in HepG2 cells. The CAT reporter plasmid p10 used for transfection experiments in Jurkat cells consists of a chimeric c-fos promoter gene fusion carrying the Gibbon ape leukaemia virus-TPA-responsive element enhancer and has been described previously (49). Dominant negative coding sequences (OH-Fos, 4H-Fos, A-Fos, A-VDP, 4H-CREB, A-CREB) were cloned as Ndel-HindIII fragments into the pRC/CMV vector (Invitrogen) modified to contain a N-terminal hemagglutinin epitope (MYPYDVPDYA) pRC/CMV566 or an N-terminal FLAG epitope (MDYKDDDK) and a new polylinker. The Ndel-HindIII fragments were obtained from the proviral expression vector pT5 in which the dominant negatives had been cloned previously (see “Proteins”).

**Stable Transfections—**Stable transfection of the murine fibroblast cell line C3H10T1/2 (ATCC number CCL226) was performed as described previously (29) using the calcium phosphate DNA precipitation method. Individually precipitates containing 200 ng of pT24 Ha-ras (30) and 600 ng of each pRC/CMV vector (Invitrogen) modified to contain a N-terminal hemagglutinin epitope (MYPYDVPDYA) pRC/CMV566 or an N-terminal FLAG epitope (MDYKDDDK) and a new polylinker. The Ndel-HindIII fragments were obtained from the proviral expression vector pT5 in which the dominant negatives had been cloned previously (see “Proteins”).

**Indirect Immunofluorescence—**HepG2 cells were cultured in 1-ml slide flasks (Nunc). Cells were fixed in 4% formaldehyde in PBS for 20 min at room temperature followed by methanol for 10 min. After blocking with 3% bovine serum albumin in PBS and 0.1% Tween 20 for 30 min at room temperature, slides were incubated with a 1:200 FLAG M2 antibody and 3% bovine serum albumin in PBS for 2 h, followed by incubation for 1 h with fluorescein isothiocyanate-conjugated rat anti-mouse antibody used at a 1:200 dilution in PBS containing 3% bovine serum albumin. After each incubation with antibodies, cells were extensively washed with PBS, 0.05% Twenn 20 for 10 min each at room temperature.

**Eukaryotic Plasmids—**The eukaryotic expression plasmid containing chicken Jun is driven by the CMV promoter and has been described elsewhere (21). The CAT reporter plasmid containing a single AP1 binding site was constructed by inserting the AP1 consensus site (126) into AP1 (46), converted to K socialism values were calculated previously (26) and G (using a ΔC of -1.4 kcal/mol °C calculated from a Tm versus ΔT plot for all of the proteins used in this study. All thermal melts were reversible. The spectra were recorded in a 0.5-cm cuvette.
colonies obtained from a group transfected with Ha-ras plus pRc/ CMV566, which is set at 1.00. The values reported (in most instances) have been calculated from the numbers obtained from multiple, independent experiments. The ratio of colony forming efficiency (CE) to focus formation efficiency (FFE) assesses the relative contribution of a decrease in CE to the observed level of FFE. CE/FFE values greater than 1.0 are considered significant.

RESULTS

Design of Acidic Amphipathic Extension That Forms a Coiled Coil with the Jun Basic Region—Previously, we showed that three bZIP basic regions from C/EBP, VBP, and GBF1, when appended onto the N-terminus of the C/EBP leucine zipper, were able to form a heterodimeric coiled coil structure with a designed acidic amphipathic protein sequence. The acidic amphipathic extension had been appended onto the N-terminus of a leucine zipper designed to preferentially interact with the C/EBP leucine zipper (Fig. 1) (26, 27). This acid amphipathic extension (4H-) of the leucine zipper created a potent DN that heterodimerized with the bZIP protein C/EBP and prevented DNA binding. We explored the generality of this method by appending the acidic amphipathic extension onto the Fos leucine zipper in an attempt to inhibit Fos:Jun DNA binding. The rationale was that the acidic extension would electrostatically mimic DNA and provide the Jun basic region with an alternate interaction surface. The Jun basic region, instead of binding in the major groove of DNA, would form a heterodimeric coiled coil structure with the acidic amphipathic protein sequence.

The above strategy was unsuccessful because the Jun basic region is different from the three basic regions examined previously (Fig. 1). There is a hydrophobic amino acid (isoleucine) in the a position immediately N-terminal of the first d position of the Jun leucine zipper, while the three previous basic regions (C/EBP, VBP, and GBF1) contained a polar amino acid (asparagine, glutamate, or cysteine) in this position. Previously, we placed an asparagine in the corresponding position of the acidic extension (4H-) to create a polar interaction in the hydrophobic interface as is seen in the leucine zipper, which also contains an N in the a position (28). In the new acidic extension (N4H-) (Fig. 1), we have replaced this polar asparagine within 4H- with a hydrophobic leucine, reasoning that the hydrophobic isoleucine of the Jun basic region would interact more favorably with a leucine than an asparagine. For simplicity, we refer to N4H-Fos as A-Fos, where A refers to acidic extension.

The New Acidic Extension of the Fos Zipper Stabilizes the Interaction with the Jun bZIP Domain 3000-fold—The thermal stability of mixtures of the Jun and Fos bZIP domains and the Jun bZIP domain with different potential dominant negatives was monitored using CD spectroscopy (Fig. 2, Table I). The dissociation constants were calculated at 37 °C because they provide information about dimerization in vivo. The Fos bZIP domain is so unstable that we were unable to determine reliably a dissociation constant. The Jun bZIP domain, however, does produce an interpretable thermal melt with a $K_d = 10^{-5}$ M (Table I). The mixture of Fos and Jun forms a heterodimer. This is demonstrated by the greater stability of the mixture (Jun + Fos) are shown with open circles. The solid line labeled Sum is what we would expect if Fos and Jun did not interact. The melts of the mixtures of Jun with the three potential dominant negatives (Jun + OH-Fos, Jun + 4H-Fos, and Jun + A-Fos) are shown with closed points. The fitted curve through each of the data sets was used to calculate $T_m$ as described previously. The $K_d$, for each mixture are shown in M (26). B, CD thermal melting curves at 222 nm of 1) A-Fos, 2) Jun, and 3) Jun + A-Fos. The solid line labeled Sum is what we would expect if Jun and A-Fos did not interact. C, CD thermal melting curves at 222 nm of 1) A-Fos, 2) VBP, and 3) VBP + A-Fos. The solid line labeled Sum is what we would expect if VBP and A-Fos did not interact.
mixture \( K_d(37) = 9 \times 10^{-8} \text{ M} \), which is greater than the sum of the individual Fos and Jun thermal melts (solid line) (Fig. 2A). The deduced \( K_d(25) \) for a Fos and Jun mixture is \( 1.3 \times 10^{-8} \text{ M} \), which is similar to the value of \( K_d(25) = 2.3 \times 10^{-8} \text{ M} \) reported earlier using fluorescence energy transfer assay (23).

We then examined the thermal stability of the Jun bZIP domain mixed with three potential dominant negatives: the Fos leucine zipper without the basic region or the Fos leucine zipper with one of two different acidic extensions appended onto the N-terminus (Fig. 2A, Table I). The mixture of Jun with Fos without a basic region (Jun + 0H-Fos) is twice as stable (\( \Delta G = -0.4 \text{ kcal/mol} \)) as the Jun + Fos mixture, indicating that the basic regions are repulsive; a similar result was seen with the C/EBP basic region (26). A surprising result was that the ellipticity at 6 °C for the Fos + Jun mixture was greater than for the mixture of Fos without the basic region (0H-Fos) and Jun. This suggests that the basic regions are helical in the absence of DNA, an observation that is not seen for C/EBP (26). The addition of the acidic amphipathic extension to the N-terminus of the Fos leucine zipper dramatically stabilizes the interaction with Jun. Using the previously described acidic extension (4H-Fos), we observe a 30-fold increase (\( \Delta G = -1.8 \text{ kcal/mol} \)) in the stability of the Jun bZIP domain. The new acidic extension containing the single Asn \( \rightarrow \) Leu change (A-Fos) is 3000-fold more stable (\( \Delta G = -4.6 \text{ kcal/mol} \)) than the Jun + 0H-Fos mixture (Fig. 2A, B, and Table I). The single amino acid change increased the heterodimer stability 2.8 kcal/mol.

The specificity of the interaction of A-Fos with additional bZIP domains was determined by CD thermal denaturation. The VBP bZIP domain and A-Fos were thermally denatured, either alone or together, and no interaction was observed (Fig. 2C). Similar results were obtained for the mixture of 4H-Fos and C/EBP (data not shown). This suggests that the acidic extension is only able to interact with the basic region if the leucine zippers themselves are physically interacting.

To determine if the new acidic extension containing the Asn \( \rightarrow \) Leu change (A-) stabilized other bZIP basic regions or was specific for a basic region containing a hydrophobic residue in the a position, we appended the new acidic sequence onto the C/EBP leucine zipper (A-C/EBP) and determined the thermal stability of mixtures with C/EBP (Table I). C/EBP interacts similarly with both acidic extensions with the following dissociation constants: \( K_d(37) = 7 \times 10^{-10} \text{ M} \) for 4H-C/EBP and \( 8 \times 10^{-10} \text{ M} \) for A-C/EBP. In the context of the C/EBP leucine zipper, the new acidic extension only contributes to a 0.1 kcal/mol increase in stability, which is negligible. These data demonstrate that the Asn \( \rightarrow \) Leu change produces a new acidic extension that interacts well with all bZIP basic regions examined but prefers to interact with basic regions containing a hydrophobic residue in the a position, e.g. the Jun basic region.

The Acidic Extension Does Not Increase the a-Helical Content of the Jun Basic Domain—A puzzling result from the thermal denaturation experiments was the amount of ellipticity at 222 nm indicative of a-helical structure seen at low temperatures when the samples are dimeric. The mixture of the Jun bZIP domain mixed with three potential dominant negatives: the Fos leucine zipper without the basic region or the Fos leucine zipper with one of two different acidic extensions appended onto the N-terminus (Fig. 2A). The deduced \( K_d(25) \) for a Fos and Jun mixture is 1.3 \( \times 10^{-8} \text{ M} \), which is similar to the value of \( K_d(25) = 2.3 \times 10^{-8} \text{ M} \) reported earlier using fluorescence energy transfer assay (23).
and Fos bZIP domains (Jun + Fos) has more helicity than the mixture of Jun bZIP and the Fos leucine zipper (Jun + 0H-Fos) (Fig. 2A). This suggests that the basic regions of Jun and Fos are helical in the absence of DNA. This result was not observed when similar experiments were done with C/EBP. The basic regions of the bZIP proteins GCN4 and C/EBP have been shown to be nonhelical in the absence of DNA and to become helical when bound to DNA (15, 16, 31). We measured the CD spectra from 200 to 250 nm of C/EBP and Fos:Jun in the absence and presence of sequence-specific DNA (Fig. 3). CD ellipticity at 222 nm is indicative of helicity. As reported earlier, we observe that the C/EBP bZIP domain shows a 44% increase in helicity with the addition of DNA (Fig. 3A) (31). In contrast, the Fos:Jun heterodimer shows only a modest 10% increase in helicity after binding sequence-specific DNA (Fig. 3B), an increase identical to that reported earlier (32). Interestingly, the mixture of Jun with a Fos zipper lacking the basic region (Jun + 0H-Fos) contains 40% less helicity than the mixture of the Fos and Jun bZIP domains (Fig. 3B). This suggests that the Jun and Fos basic regions are largely helical in the absence of DNA, unlike the C/EBP basic region, although the basic regions are repulsive.

Inhibition of AP1 DNA Binding—Gel shift experiments were undertaken to examine the number of molar equivalents of A-Fos that would be needed to inhibit the DNA binding of a mixture of Fos and Jun. Published data indicate that a Fos:Jun heterodimer binds DNA with a $K_{d}$ of $2 \times 10^{-10}$ M (33). CD experiments presented in Table I indicate that A-Fos heterodimerizes with Jun with a $K_{d}$ of $2.8 \times 10^{-12}$ M. Therefore, an equimolar mixture of Fos, Jun, and A-Fos should prevent Fos:Jun heterodimers from binding DNA because of the preferred formation of the Jun:A-Fos heterodimer. Fos, Jun, or a Fos + Jun mixture was incubated with a labeled 24-base pair oligonucleotide containing a single AP1 site and tested for DNA binding using a gel shift assay. Fig. 4A shows that Fos does not bind AP1 DNA (lane 1) but that Jun:Jun homodimers bind slightly (lane 2) and Fos:Jun heterodimers bind well (lane 3). One molar equivalent of A-Fos is able to totally inhibit Fos:Jun DNA binding (lane 6). The Fos leucine zipper without the acidic extension (0H-Fos) at equimolar concentrations does not inhibit Fos:Jun binding (lane 4), while the previously described acidic extension (4H-Fos) inhibits Fos:Jun binding only partially as indicated by the amount of free probe remaining in the reaction. These data demonstrate that the mutation Asn → Leu has a dramatic effect on the ability of a DN to prevent AP1 DNA binding. At equimolar concentrations, 0H-Fos and 4H-Fos are not expected to inhibit Fos:Jun DNA binding, because they heterodimerize with Jun with a lower affinity than Fos:Jun bound to DNA. Indeed, Jun:0H-Fos shows a $K_{d}(25) = 8.5 \times 10^{-7}$ M and Jun:4H-Fos shows a $K_{d}(25) = 4.2 \times 10^{-10}$ M.

To examine the specificity of the A-Fos ability to inhibit a Fos:Jun mixture from binding DNA, we undertook two controls. The first control was used to determine whether the leucine zippers of 4H-Fos and A-Fos are critical for the inhibition of Fos:Jun DNA binding. Chimeric proteins were generated where the Fos leucine zipper was replaced with the VBP or the CREB leucine zipper. Fos:Jun DNA binding is not inhibited

![Inhibition of AP1 DNA Binding](image-url)
Inhibition of AP1 DNA Binding

Inhibition of AP1 Transactivation—A transient transfection assay in a human hepatoma cell line (HepG2) was employed to examine the dominant negative properties of A-Fos. HepG2 cells were co-transfected with the Jun transactivator and a CAT reporter gene containing a single AP1 binding site. Jun is able to transactivate this promoter 10-fold (Fig. 5A). Four different potential DNs were tested for their ability to inhibit Jun transactivation at a 1:1 molar ratio, an experimental condition where we tried to avoid overexpression of the DN. These DNs are the Fos bZIP domain with the transactivation domain deleted (bZIP-Fos), the Fos leucine zipper (0H-Fos), and the Fos leucine zipper with the two acidic amphipathic extensions (4H- and A-) appended onto the N-terminus. Neither the Fos bZIP domain nor the Fos leucine zipper, two possible DNs that could occur by the simple deletion within the fos gene, were able to inhibit transactivation under the experimental conditions used. 4H-Fos and A-Fos inhibited Jun transactivation over 80%. Complete inhibition is observed when a 3:1 molar ratio of the A-Fos to Jun transactivator is used. The expression of A-Fos was checked by Western blot (Fig. 5B) using the N-terminal FLAG epitope present on each of the DN proteins. The AP1 complex is supershifted by a Jun family antibody. The ability of A-Fos to inhibit the DNA binding of a native AP1 complex was determined (Fig. 4C). Purified AP1 protein complex, isolated from T cells, was bound to an AP1-specific DNA sequence (34). A-Fos was able to totally inhibit DNA binding (lane 2), while the same acidic extension appended to the VBP leucine zipper was not able to inhibit DNA binding (lane 3). The composition of the AP1 purified complex was examined by performing an AP1 DNA binding assay in the presence of supershifting antibodies directed against Jun family proteins (lane 4). The AP1 complex is supershifted by a Jun but not an NF-κB antibody. These results demonstrate two points: A-Fos is able to inhibit the DNA binding of a native AP1 complex, and the inhibition of DNA binding caused by A-Fos is leucine zipper-dependent (Fig. 3C, lanes 3 and 4).

Inhibition of AP1 DNA Binding—A transient transfection assay in a human hepatoma cell line (HepG2) was employed to examine the dominant negative properties of A-Fos. HepG2 cells were co-transfected with the Jun transactivator and a CAT reporter gene containing a single AP1 binding site. Jun is able to transactivate this promoter 10-fold (Fig. 5A). Four different potential DNs were tested for their ability to inhibit Jun transactivation at a 1:1 molar ratio, an experimental condition where we tried to avoid overexpression of the DN. These DNs are the Fos bZIP domain with the transactivation domain deleted (bZIP-Fos), the Fos leucine zipper (0H-Fos), and the Fos leucine zipper with the two acidic amphipathic extensions (4H- and A-) appended onto the N-terminus. Neither the Fos bZIP domain nor the Fos leucine zipper, two possible DNs that could occur by the simple deletion within the fos gene, were able to inhibit transactivation under the experimental conditions used. 4H-Fos and A-Fos inhibited Jun transactivation over 80%. Complete inhibition is observed when a 3:1 molar ratio of the A-Fos to Jun transactivator is used. The expression of the different DN proteins was checked by Western blot (Fig. 5B) using the N-terminal FLAG epitope present on each of the DN proteins. The AP1 complex is supershifted by a Jun family antibody. The ability of A-Fos to inhibit the DNA binding of a native AP1 complex was determined (Fig. 4C). Purified AP1 protein complex, isolated from T cells, was bound to an AP1-specific DNA sequence (34). A-Fos was able to totally inhibit DNA binding (lane 2), while the same acidic extension appended to the VBP leucine zipper was not able to inhibit DNA binding (lane 3). The composition of the AP1 purified complex was examined by performing an AP1 DNA binding assay in the presence of supershifting antibodies directed against Jun family proteins (lane 4). The AP1 complex is supershifted by a Jun but not an NF-κB antibody. These results demonstrate two points: A-Fos is able to inhibit the DNA binding of a native AP1 complex, and the inhibition of DNA binding caused by A-Fos is leucine zipper-dependent (Fig. 3C, lanes 3 and 4).

To investigate whether A-Fos inhibition of Jun transactivation is dependent on the Fos leucine zipper (Fig. 5A), chimeric DNs were generated where the Fos leucine zipper was replaced by three different leucine zippers (C/EBP, CREB, or VBP). These chimeric DNs are not able to inhibit Jun transactivation, indicating that A-Fos is acting in a leucine zipper-specific manner. The reporter gene contains a single CREB cis element, and the transactivator is C/EBPα. C/EBPα is able to transactivate the CREB-containing promoter and is not inhibited by 4H-Fos or A-Fos. Transfections were carried out using 10 μg of the reporter containing the CREB cis element, 0.3 μg of mouse sarcoma virus C/EBPα, and 0.3 μg of 4H-Fos or 0.3 μg of A-Fos. 4H-Fos inhibits TPA-induced T cell activation. Jurkat cells were transfected with 8 μg of the reporter plasmid p10 and either 0.5 or 2 μg of 4H-Fos expression vector and induced with phorbol 12-myristate 13-acetate and phytohemagglutinin for 18 h. CAT activity was measured and expressed as fold activation ± S.D. relative to the activity of the reporter plasmid alone (noninduced).
Inhibition of AP1 DNA Binding

To examine whether A-Fos is acting nonspecifically by inactivating transcription, we determined whether A-Fos inhibited the transactivation of C/EBPα, another bZIP transactivator. HepG2 cells were transfected with a CAT reporter gene containing 1) a single C/EBP binding site in the promoter and C/EBPα alone, 2) C/EBPα plus 4H-Fos, or 3) C/EBPα plus A-Fos (Fig. 5D). C/EBPα is able to activate a single C/EBP cis element 7-fold. Either acidic extension appended onto the N-terminus of the Fos zipper does not inhibit C/EBPα transactivation, demonstrating that the dominant negative to AP1 does not inhibit the function of other bZIP proteins.

The human Jurkat T cell line was used as a model to examine the effect of the Fos dominant negative (4H-Fos) on TPA-induced T cell activation (Fig. 5E). Incubation of Jurkat cells with phytohemagglutinin and phorbol 12-myristate 13-acetate phorbol ester (equivalent to TPA) results in the production of interleukin-2. The interleukin-2 promoter contains a TPA-responsive element that binds AP1 proteins and is a major target for the phorbol ester response. Jurkat cells were transfected with a CAT reporter gene containing three TPA-responsive elements. The addition of TPA results in a 10-fold activation of the reporter gene, and the co-transfection of 4H-Fos inhibits the TPA activation in a dose-dependent fashion. This result demonstrates that 4H-Fos is able to inhibit the transcriptional activity of a native AP1 complex in T cells.

**DN to AP1 Inhibits Ha-ras-mediated Cellular Transformation More than Cell Growth—**AP1 is an immediate early protein complex that plays an important role in the initiation of cellular growth (3). The activity of AP1 also is a critical downstream mediator of the proliferative effects of several oncogenes, most notably members of the Ras family (35–37). To investigate the possibility that DN to AP1 could be used to dissect further the role of AP1 in cellular growth and transformation, C57BL/10T strain mouse fibroblasts were stably transfected with the human Ha-ras oncogene and a 3-fold molar excess of vector DNA, bZIP-Fos, 0H-Fos, or A-Fos. The transfected cells were plated as described under "Experimental Procedures" to assay for focus formation as well as to assay for the ability of DN expressing cells to produce stable, G418^R colonies (Table II). In most cases, the results from multiple, independent experiments were averaged, and the focus forming and colony forming efficiencies are expressed relative to the appropriate Ha-ras control. The results show that all three DN constructs inhibit both C57BL/10T strain cell growth and Ha-ras-mediated cellular transformation. The Fos bZIP domain lacking a transactivation domain, the type of DN previously used (20), reduced foci formation to 59% of the Ha-ras control while only inhibiting colony formation to 78% of controls. Using the same concentration of input DNA, 0H-Fos inhibits foci formation to 40%, while the acidic extension (A-Fos) decreases foci formation to 30% and inhibits colony formation to only 63%. Interestingly, when the average reduction in CE is expressed relative to the average inhibition in FFE, the A-Fos construct was shown to inhibit transformation 2-fold over the level anticipated from the observed impact of this protein on C57BL/10T1/2 cell growth. Reductions in focus formation similar to those seen with the DN to AP1 were not observed when 4H-CREB and 4H-C/EBP were tested in Ha-ras transformation assays, indicating that the inhibition in foci formation is dependent on the Fos leucine zipper (Table II). We take these results to mean that A-Fos has the potential to be used as a highly specific DN to experimentally separate the various biological activities of AP1 in mammalian cells.

**DISCUSSION**

We have generated a DN protein termed A-Fos that inhibits the DNA binding of AP1 in an equimolar competition. AP1 is a heterodimer composed of two bZIP proteins, a Fos family member and a Jun family member. A-Fos contains an acidic amphipathic protein sequence appended onto the N-terminus of the Fos leucine zipper, and the acidic extension physically interacts with the Jun basic region. The interaction of the Jun basic region with the acidic extension extends the leucine zipper dimerization interface into the basic region, thus preventing the basic region from binding DNA (Fig. 6). The acidic protein sequence stabilizes the interaction of the Fos zipper with the Jun bZIP domain 4.6 kcal/mol or 3000-fold. This increase in heterodimer stability makes this DN an effective competitor in a stoichiometric competition. Gel shift experiments indicate that A-Fos is indeed able to inhibit Fos:Jun DNA binding in an equimolar competition experiment. Neither the Fos leucine zipper alone nor a previously described acidic extension (4H-) appended onto the N-terminus of the Fos zipper was able to inhibit Fos:Jun DNA binding, demonstrating that the newly described acidic extension is critical for the function of the A-Fos DN. The specificity of the inhibition of DNA binding was assayed by replacing the Fos leucine zipper with sequences obtained from two heterologous zipper proteins, C/EBP and VBP. These proteins were not able to inhibit Fos:Jun DNA binding, indicating that the specificity of the DN is derived from the leucine zipper and the stability of the complex from the acidic extension.

The ability of this newly designed DN (A-Fos) to inhibit the biological function of AP1 was tested in a variety of assays. A co-transfection assay using human hepatoma cells (HepG2) shows that A-Fos is able to inhibit Jun-dependent transactivation of a promoter containing a single AP1 cis element when

---

**Table II**

Inhibition of cellular growth and transformation by dominant negatives to AP1

| Number of foci | FFE | Number of G418^R colonies | CE | CE/FFE |
|----------------|-----|--------------------------|----|--------|
|                | Exp. 1 | Exp. 2 | Exp. 3 | Exp. 4 | Exp. 1 | Exp. 2 |    |    |
| Ha-ras         | 207    | 134    | 123    | 156    | 1.08 | 0.97 | 0.59 | 1.20 |
| Ha-ras + vector | 152    |        |        |        | 1.00 | 0.97 | 0.59 | 1.20 |
| Ha-ras + bZIP-Fos | 86     | 80     | 0.59 |        | 0.59 | 0.59 | 0.59 | 0.59 |
| Ha-ras + 0H-Fos | 75     | 54     | 42     | 0.40 | 288  | 233  | 0.58 | 1.45 |
| Ha-ras + A-Fos  | 53     | 41     | 35     | 0.30 | 314  | 249  | 0.63 | 2.10 |
| Ha-ras         | 134    |        |        |        | 1.00 | 0.97 | 0.59 | 1.20 |
| Ha-ras + 4H-CREB | 97    |        |        |        | 0.72 | 0.72 | 0.72 | 0.72 |
| Ha-ras + 4H-C/EBP | 161    |        |        |        | 1.20 | 1.20 | 1.20 | 1.20 |

* Exp., experiment.
equal molar quantities of A-Fos plasmid DNA were added to the transfection, Jun-dependent transactivation was decreased to 20% of controls. When a 3-fold molar excess of DN was added, Jun-dependent transactivation was totally abolished. The specificity of the inhibition was tested using two separate approaches. The inhibition of Jun-dependent transactivation was tested by appending the acidic extension onto several other leucine zipper sequences that do not dimerize with either the Fos or Jun leucine zippers. These potential DNs did not inhibit Jun-dependent transactivation. The second approach was to test whether A-Fos inhibits transactivation by the bZIP protein C/EBPα. The transactivation of C/EBPα is not inhibited by the A-Fos DN, demonstrating that A-Fos only inhibits leucine zippers with which it can physically interact.

The human Jurkat T cell line was used as a model to examine the effect of the Fos dominant negative on TPA-induced T cell activation. Incubation of Jurkat cells with phytethylamglutinin and TPA results in the production of interleukin-2. We showed that 4H-Fos is able to inhibit the transcriptional activity of a native AP1 complex in T cells, implying that it can be used as a reagent to study the the cascade of events leading to the the activation of the interleukin-2 gene.

A cellular transformation assay in C1H10T1/2 cells was utilized to explore whether A-Fos could inhibit Ras-dependent transformation. As an immediate early transcription complex, AP1 has been shown to be important for the initiation of cell growth (1, 38). In addition, the oncogenicity of retrovirally encoded variants of the c-Fos and c-Jun proteins has demonstrated a role for AP1 activity in cellular transformation. As a target for positive regulation by the mitogen-activated protein genes regulated by AP1.

A DN described in this manuscript, acts in a novel fashion; it heterodimerizes with endogenous AP1 family members, which inhibits DNA binding, thereby inhibiting transactivation and transformation. A-Fos, in both transactivation and transformation assays is more effective than bZIP-Fos or the Fos leucine zipper, the type of DNs used previously.

An additional advantage of the DN strategy presented here is the ability to explore repressive effects of a transcription factor. In different promoter contexts, some DNA-binding proteins can be either activators or repressors (41). The typical DN consisting of the bZIP domain could inhibit transactivation but not repression because the DNA site would remain occupied. Thus, previously described DNs are valuable for exploring the transactivation properties of a transcription factor but not the repression properties. The DNs described here should reveal any repressive properties of the AP1 complex in certain cell types and physiological conditions (40).

The experimental problem with these truncated proteins consisting of only the bZIP domain is the difficulty of demonstrating the mode of action. The DN to AP1 we have synthesized and characterized in this study forms heterodimers with Jun and prevents the normal AP1 family complex from binding DNA, creating a situation akin to ablating Jun family members' function in a cell. The inhibition of DNA binding should allow the demonstration in biological assays of the absence of DNA site occupancy using an in vivo footprint assay (25) and allow for the design of future experiments to identify target genes regulated by AP1.

Acknowledgments—We thank C. Peterson and J. Moitra for comments on the manuscript, M. Birrer and N. Colburn for insightful conversations, and Claude Klee for support and encouragement.

REFERENCES
1. Herschman, H. R. (1991) Annu. Rev. Biochem. 60, 281–319
2. Ransone, L., and Verma, I. (1990) Annu. Rev. Cell Biol. 6, 539–557
3. Angel, P., and Karin, M. (1991) Biochim. Biophys. Acta. 1072, 129–157
4. Cohen, D. R., and Curran, T. (1988) Mol. Cell. Biol. 8, 2063–2069
5. Zerial, M., Toschi, L., Ryseck, R. P., Schuermann, M., Muller, R., and Bravo, R. (1989) EMBO J. 8, 805–813
6. Nishina, H., Sato, H., Suzuki, T., Sato, M., and Iba, H. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3619–3623
7. Bohmann, D., Roos, T. J., Admon, A., Nishimura, T., Vogt, P. K., and Tjian, R. (1987) Science 236, 1386–1392
8. Mak, Y., Roos, T. J., Davies, C., Starbuck, M., and Vogt, P. K. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2848–2852
9. Hirai, S. I., Ryseck, R. P., Mechina, F., Bravo, R., and Yaniv, M. (1989) EMBO J. 8, 1433–1439
10. Ryder, K., Lanahan, A., Perez-Albuerne, E., and Nathans, D. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1500–1503
11. Baezeman, A., and Vinson, C. R. (1993) Curr. Opin. Genet. Dev. 3, 278–285
12. Landschulz, W. H., Johnson, P. F., and McKnight, S. L. (1988) Science 240, 1759–1764
13. Harbury, P. B., Zhang, T., Kim, P. S., and Alber, T. (1993) Science 262, 1401–1407
14. Vinson, C. R., Sigler, P. B., and McKnight, S. L. (1989) Science 246, 911–916
15. O’Neil, K., Hoess, R., and DeGrado, W. (1990) Science 246, 774–778
16. Shuman, J. D., Vinson, C. R., and McKnight, S. L. (1990) Science 249, 771–774
Inhibition of AP1 DNA Binding

17. Akira, S., Ishihiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T., and Kishimoto, T. (1990) EMBO J. 9, 1897–1906
18. Lloyd, A., Yancheva, N., and Wasylyk, B. (1991) Nature 352, 635–638
19. Brown, P. H., Alani, R., Preis, L. H., Szabo, K., and Birrer, M. J. (1993) Oncogene 8, 877–886
20. Wick, M., Lucibell, F., and Muller, R. (1992) Oncogene 7, 859–867
21. Brown, P., Chen, T., and Birrer, M. (1993) Oncogene 8, 791–799
22. Ham, J., Babij, C., Whitfield, J., Pfarr, C. M., Lallemand, D., Yaniv, M., and Rubin, L. L. (1995) Neuron 14, 927–939
23. Patel, L. R., Curran, T., and Kerppola, T. K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7360–7364
24. Herskowitz, I. (1987) Nature 329, 219–222
25. Mueller, P., and Wold, B. (1989) Science 246, 780–786
26. Krylov, D., Olive, M., and Vinson, C. (1995) EMBO J. 14, 5329–5337
27. Vinson, C. R., Hai, T. W., and Boyd, S. M. (1993) Genes & Dev. 7, 1047–1058
28. O’Shea, E. K., Klemm, J. D., Kim, P. S., and Alber, T. (1991) Science 254, 539–544
29. Taparowsky, E. J., Heaney, M. L., and Parsons, J. T. (1987) Cancer Res. 47, 4125–4129
30. Taparowsky, E., Suard, Y., Fasano, O., Shimizu, K., Goldfarb, M., and Wigler, M. (1982) Nature 300, 762–765
31. O’Neill, K. T., Shuman, J. D., Ampe, C., and DeGrado, W. F. (1991) Biochemistry 30, 9030–9034
32. Patel, L., Abate, C., and Curran, T. (1990) Nature 347, 572–575
33. Smeal, T., Angel, P., Meek, J., and Karin, M. (1989) Genes & Dev. 3, 2091–2100
34. Gardner, K., Moore, T. C., Davis-Smyth, T., Krutzsch, H., and Levens, D. (1994) J. Biol. Chem. 269, 32963–32971
35. Smeal, T., Binetruy, B., Mercola, D. A., Birrer, M., and Karin, M. (1991) Nature 354, 494–496
36. Suzuki, T., Murakami, M., Onai, N., Fukuda, E., Hashimoto, Y., Sonobe, M. H., Kameda, T., Ichinose, M., Miki, K., and Iba, H. (1994) J. Virol. 68, 3527–3535
37. Johnson, R., Spiegelman, B., Hanahan, D., and Wisdom, R. (1996) Mol. Cell. Biol. 16, 4504–4511
38. Lai, L. F., and Nathans, D. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1182–1186
39. Pulverer, B. J., Kryiakiski, J. M., Avruch, J., Nikolakaki, E., and Woodgett, J. R. (1991) Nature 353, 670–674
40. Pfarr, C. M., Meeha, F., Spyrou, G., Lallemand, D., Carillo, S., and Yaniv, M. (1994) Cell 76, 747–760
41. Clark, A. R., and Docherty, K. (1993) Biochem. J. 296, 521–541
42. Descombes, P., and Schibler, U. (1991) Cell 67, 569–579
43. Foulkes, N. S., Borrelli, E., and Sassone-Corsi, P. (1991) Cell 64, 739–749
44. Yin, J., Wallach, J., Vecchio, M. D., Wilder, E. L., Zhou, H., Quinn, W., and Tully, T. (1994) Cell 79, 49–58
45. Powers, C., Krutzsch, H., and Gardner, K. (1996) J. Biol. Chem. 271, 20089–20095
46. Krylov, D., Mikhailenko, I., and Vinson, C. R. (1994) EMBO J. 13, 2849–2861
47. Olive, M., Williams, S., Dezan, C., Johnson, P., and Vinson, C. (1996) J. Biol. Chem. 271, 2040–2047
48. Wang, Y., Feinberg, M., Hosking, J. B., Bogerd, H., and Greene, W. C. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 9733–9737
49. Farina, A. R., Davis-Smyth, T., Gardner, K., and Levens, D. (1993) J. Biol. Chem. 268, 26466–26475