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Repression of IL-2 Promoter Activity by the Novel Basic Leucine Zipper p21^{SNFT} Protein[1,2]

Milena Iacobelli,* William Wachsman,† and Kathleen L. McGuire[3]*

IL-2 is the major autocrine and paracrine growth factor produced by T cells upon T cell stimulation. The inducible expression of IL-2 is highly regulated by multiple transcription factors, particularly AP-1, which coordinately activate the promoter. Described here is the ability of the novel basic leucine zipper protein p21^{SNFT} to repress AP-1 activity and IL-2 transcription. A detailed analysis of the repression by p21^{SNFT} repression on the IL-2 promoter distal NF-AT/AP-1 site demonstrates that it can bind DNA with NF-AT and Jun, strongly suggesting that it represses NF-AT/AP-1 activity by competing with Fos proteins for Jun dimerization. The importance of this repression is that p21^{SNFT} inhibits the *trans*-activation potential of protein complexes that contain Jun, thereby demonstrating an additional level of control for the highly regulated, ubiquitous AP-1 transcription factor and the IL-2 gene. ***The Journal of Immunology,*** **2000, 165: 860–868.**

Activated Th cells of the immune system produce high levels of the IL-2 cytokine when properly stimulated through both the TCR and the CD28 costimulatory molecule (1, 2). IL-2 production is indicative of T cell activation and is the major autocrine and paracrine growth factor for T cells. While not produced by normal, quiescent T cells, the IL-2 gene rapidly becomes transcriptionally active upon stimulation. IL-2 mRNA production is detectable within 40 min, peaks at 4–6 h, and returns to background levels by 24 h (2, 3). Although control of IL-2 protein synthesis occurs at many levels, it is principally regulated at the level of transcription.

Although there is evidence that sequences outside of the ~300-bp human IL-2 promoter contribute to transcriptional control in vivo, this region is primarily responsible for inducible expression of the gene (4). The promoter contains a variety of binding sites for known activating transcription factors, such as NF-AT, NF-κB, AP-1, and OCT,[4] as well as the inhibitory protein NIL-2 (2, 5). All four of the activating factors have been shown to have multiple binding sites within the promoter. In addition, the proximal OCT (6, 7), the CD28 response element (8–10), and the distal NF-AT (11, 12) elements have all been shown to work with adjacent AP-1 or AP-1-like sites for binding and/or functional activity. The significance of AP-1 for the activity of this promoter has been demonstrated for the positive regulation of IL-2 expression, as well as in anergic T cells, where a lack of AP-1 activity is thought to be responsible for the inhibition of IL-2 production (13–17).

The AP-1 transcription factor consists of heterodimers between the Fos and Jun families of proteins through their basic leucine zipper (bZIP) domains. The expression and *trans*-activation potential of the AP-1 transcription factor are highly dependent on the activation of stress and mitogenic signal transduction cascades (18). Activation of the JNK and MEK MAP kinase pathways not only controls the overall levels of Fos and Jun proteins, but also leads to the proper phosphorylation states of these proteins, which are required for transcriptional activity (18). Although c-Jun and c-Fos dimers are considered to be the classical AP-1 transcription factor, a variety of dimer combinations can exist among Fos, Jun, and CREB/ATF family members, lending specificity of a particular dimer pair to an AP-1 or AP-1-like enhancer (TRE). Dimers of Jun and Fos or CREB/ATF have been shown to regulate the expression of a variety of immunologically important genes involved in T cell activation and cytokine expression (19).

Additional proteins have been described that are capable of dimerizing with Jun but that do not belong to either the Fos or CREB/ATF family. These rat proteins are called Jun dimerization proteins 1 and 2 (JDP1 and JDP-2) and are capable of repressing the expression of an AP-1-regulated reporter construct (20). JDP-1, like the closely related human B-ATF protein, can bind with Jun to a TRE sequence (20, 21). JDP-1 and B-ATF are also closely related to the Merek's disease virus EcoQ protein (MEQ), another bZIP protein capable of Jun dimerization but whose interactions with Jun lead to viral gene transcription (22). It currently is not clear what the physiological relevance is of Jun interactions with proteins such as B-ATF, JDP1, and JDP-2, because their ability to dimerize with Jun has not been shown to influence the transcription of any cellular gene.

Recently, a novel human protein named p21^{SNFT} (for 21-kDa small nuclear factor isolated from T cells) was identified that dimerizes with Jun proteins and represses AP-1 activity (W. Klump and W. Wachsman, manuscript in preparation). The biochemical characteristics of p21^{SNFT} are similar to the known activities of the JDP proteins, and it shares high amino acid identity

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[2] The GenBank accession number for the p21^{SNFT} coding sequence is AF255346.

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[4] Abbreviations used in this paper: OCT, octomer binding protein; NIL-2, negative regulator of IL-2; bZIP, basic leucine zipper; JNK, Jun N-terminal kinase; MEK, mitogen-activated kinase-extracellular regulated kinase kinase; MEKK, MEK kinase; CREB, cAMP response element binding protein; ATF, activating transcription factor; p21^{SNFT}, 21-kDa small nuclear factor isolated from T cells; TRE, 12-*O*-tetradecanoate-13-acetate response element; JDP, Jun dimerization protein; MEQ, Merek's disease virus EcoQ protein; HTLV-I, human T cell leukemia virus type I; LTR, long terminal repeat; RSV, Rous sarcoma virus; FRK, Fos-regulating kinase; PKA, protein kinase A; CAT, chloramphenicol acetyltransferase.

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(69%) to rat JDP-1, suggesting that $p21^{SNFT}$ may be its human homologue. Due to the properties of $p21^{SNFT}$ and the function of AP-1 in IL-2 gene expression, the involvement of this factor in the transcriptional regulation of IL-2 was investigated.

The work shows that $p21^{SNFT}$ significantly and specifically down-regulates IL-2 promoter activity and endogenous IL-2 production by Jurkat cells. The ability of $p21^{SNFT}$ to repress the IL-2 promoter occurs though multiple IL-2 enhancers that functionally require AP-1. $p21^{SNFT}$ is shown to bind TRE sequences with Jun, to the exclusion of Fos, and to bind to the IL-2 promoter distal NF-AT/AP-1 binding site with NF-AT and Jun in complexes that also do not contain Fos. Elevated concentrations of $p21^{SNFT}$ relative to c-Fos result in a decrease in Fos/Jun dimer formation and subsequent AP-1 activity. Therefore, the transcriptional inhibition by $p21^{SNFT}$ appears to be a consequence of the ability of $p21^{SNFT}$ to interact with Jun's bZIP domain, thereby reducing Fos/Jun associations.

Materials and Methods

Transfections

Jurkat cells were transfected as previously described (8) with 5 μg of luciferase or chloramphenicol acetyltransferase (CAT) reporter construct, 1–5 μg of pCI/SNFT expression construct or empty vector (pCI), and 5 μg of a human growth hormone-expressing construct for internal normalization of transfection efficiency and cell survival. Cells were stimulated with 10 ng/ml PMA, 1.5–3 μM ionomycin, and a 1/2500 dilution of mAb 9.3 ascites where indicated 20 h posttransfection and were harvested 20 h poststimulation. Luciferase, CAT, and growth hormone assays were performed as previously described (9). Enhancer studies were conducted using the following constructs: 1) three repeats of the human IL-2 CD28RE/AP-1 sequence cloned upstream of a human T cell leukemia virus type I (HTLV-I) long terminal repeat (LTR) minimal promoter, 2 and 3) three repeats of the distal NF-AT/AP-1 and the proximal AP-1/OCT sequence cloned upstream of an IL-2 minimal promoter, and 4) two repeats of the IL-1β promoter NF-κB (23) site upstream of a c-*fos* minimal promoter. pSV2Fos was used as a human c-*fos* expression construct in the titration studies. Stable cell lines were generated by electroporating 1×10^7 Jurkat cells with 10 μg of linearized pCI (for J-CI-1 and J-CI-2 lines) or pCI/SNFT (for J-SNFT-1 and J-SNFT-2 lines). Cells were electroporated in a 0.5-ml volume of RPMI and L-glutamine in a 0.4-cm cuvette at 250 V and 960 μF. After the electrical pulse was delivered, cells were incubated on ice for 10 min, resuspended in 10 ml of medium, and cultured for 2 days. Transformants were then selected with 800 μg/ml G418 (Life Technologies, Gaithersburg, MD) for 2 wk and were subsequently maintained in medium containing 500 μg/ml G418.

Immunoprecipitations

J-CI-1, J-CI-2, J-SNFT-1, and J-SNFT-2 cells (3×10^7) were metabolically labeled with 46 μCi/ml [^{35}S]methionine (New England Nuclear, Boston, MA) in RPMI devoid of methionine and cysteine (Sigma, St. Louis, MO) containing 10% dialyzed FCS and 300 g/l L-glutamine. The cells were allowed to incorporate the radiolabeled amino acid for 5 h before being washed with PBS and resuspended in normal culture medium containing PMA (10 ng/ml) and ionomycin (1.5 μM). After 4 h, cells were harvested and lysed in 1 ml of RIPA buffer (300 mM NaCl, 100 mM EDTA, 20 mM Tris (pH 8.0), 2% Triton X, 0.02% deoxycholate, and 0.002% SDS). Chromosomal DNA was sheared by passing 6–10 times through an 18-gauge needle. Total lysate was precleared with the addition of 1 μg of rabbit IgG and 50 μl protein A-Sepharose (Pharmacia, Piscataway, NJ) and was incubated overnight with continuous agitation at 4°C. The cleared lysate was transferred to a fresh tube containing 30 μl of protein A-Sepharose and 10 μl of $p21^{SNFT}$ antiserum and was incubated with continuous agitation for 36 h at 4°C. The mixture was spun down, and the pellets were washed three times with 1 ml of wash buffer (TBS/1× RIPA, 3/1) before boiling and loading eluate onto a 12% gel for SDS-PAGE. ^{14}C-labeled protein markers (New England Nuclear) were used to confirm the migration of $p21^{SNFT}$. The gel was dried and exposed to film at 80°C.

Northern analysis

The Jurkat stable cell lines were stimulated for 4 h with 10 ng/ml PMA and 1.5 μM ionomycin to determine relative IL-2 mRNA expression by Northern analysis (24). Total RNA was prepared from 1×10^7 cells using RNA-STAT-60 (Tel-Test, Friendswood, TX) and was probed with a radioactively labeled IL-2 probe. The filter was later probed with a TCRβ probe to control for RNA loading.

Cytokine expression

The J-CI-1, J-CI-2, J-SNFT-1, and J-SNFT-2 stable cell lines were resuspended at 2.5×10^5 cells/ml in culture medium and stimulated with 10 ng/ml PMA and 1.5 μM ionomycin with or without mAb 9.3 at a 1/2500 dilution (CD28 stimulation). Cells were incubated for 4 days, and culture supernatants were harvested and tested for IL-2 and GM-CSF levels by ELISA (BioSource International, Camarillo, CA) according to the manufacturer's directions.

Electromobility shift assays

Nuclear extracts were isolated from Jurkat cells and used in the EMSA as previously described (10) except that 5 μg of nuclear extract and 0.5–1 μg of poly(dI-dC) were used. Molar equivalents of purified NF-AT, Jun, Fos, and GST were used (20–100 ng of each protein was used per sample). Molar equivalents of purified GST-SNFT was used at one of two concentrations that equaled a 1 or 5× molar ratio in relation to the other purified proteins. Where indicated, 1 μl of $p21^{SNFT}$-specific antiserum, raised in rabbits against the GST-SNFT protein (W. Klump and W. Wachsman, manuscript in preparation), was added to the reaction before incubation. The Jun- and Fos-specific antisera were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Jun-specific antiserum was a cocktail of antisera for pan-Jun (D), JunB (N-17), c-Jun (N), and JunD (329). The Fos-specific antiserum was a cocktail of antisera for Fra-1 (R-20), FosB (102), and c-Fos (4). After incubation, 20,000 cpm of [^{32}P]dATP end-labeled probe was added to the reaction and was allowed to incubate for an additional 15 min at room temperature. The samples were loaded onto a 6.6% native acrylamide gel and run at 175 V in 0.5× TBE running buffer for 5.5 h. Gels were dried and exposed to XAR5 film (Eastman Kodak, Rochester, NY) at −80°C. The sequences of the probes used were: CD28RE/AP-1 (IL-2 promoter), 5′-gatcCAGAAATTCCAAAGAGTCA TCACAgatc-3′; AP-1/OCT (IL-2 promoter), 5′-gctagcTGTGTAATTATG TAAAACtgt-3′; NF-AT/AP-1 (IL-2 promoter), 5′-gatcGGAGGAAAAACT GTTTCATACAG-3′; NF-κB (IL-2 promoter), 5′-ACAAAGAGGCTTTT CACCTACATC-3′; and TRE (consensus), 5′-GATCCGGCTGACTC ATCA-3′. The CD28RE/AP-1, NF-AT/AP-1, and AP-1/OCT probe sequences are identical with those tested in the functional studies shown in Fig. 3.

Isolation of bacterial fusion proteins

GST and GST-SNFT bacterial expression constructs were transformed into the DH5α *Escherichia coli* strain. Cells were cultured in 1 liter of Luria-Bertoni broth in a 37°C shaker until cells reached an OD_{600} of 0.6. Cells were induced to express the GST proteins with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) overnight at 30°C, then were lysed by incubation in 20 ml of lysis buffer (50 mM Tris (pH 7.9), 12.5 mM $MgCl_2$, 1 mM EDTA, 100 mM KCl, 1% Triton X-100, 20 μg/ml PMSF, 1 μg/ml pepstatin A, and leupeptin), sonicated, and spun down to remove cellular debris. The lysate was allowed to mix with glutathione-conjugated agarose beads for 2 h at 4°C with gentle agitation. The beads were washed twice with 20 ml of cold buffer I (50 mM Tris (pH 7.9), 1 M NaCl, 0.3% 2-ME, 20 μg/ml PMSF, 1 μg/ml pepstatin A, and leupeptin) and twice with 20 ml of cold buffer II (PBS, 1% Triton X-100, 0.3% β-ME, 20 μg/ml PMSF, 1 μg/ml pepstatin A, and leupeptin). Proteins were eluted by resuspending the beads in 3 column volumes of lysis buffer containing 15 mM free glutathione and 20% glycerol. Proteins were dialyzed in dialysis buffer (20 mM HEPES (pH 7.4), 1 mM DTT, 100 mM NaCl, 2 mM EDTA, 20% glycerol, and 0.01% azide) and stored at −80°C. The pNF-ATpXS (1–297) construct, which was provided by Anjana Rao (25), encodes a histidine-tagged murine NF-ATp DNA binding domain (aa 398–694). His-NF-AT was expressed in DH5α cells and was purified using the Expressionist II Kit (Qiagen, Chatsworth, CA). The purified His-NF-AT was dialyzed in dialysis buffer and stored at −80°C. Protein concentrations were determination using the Bio-Rad Protein Assay System (Hercules, CA). Purified histidine-tagged Jun (aa 187–334) and Fos (aa 139–380) proteins were provided by Tom Kerppola and were described previously (26).

Western analysis

Whole cell extracts were made from Jurkat cells stimulated with PMA and ionomycin for 0 and 30 min and 1, 2, 4, 8, and 24 h and from cells stimulated for 4 h with PMA alone or PMA, ionomycin, and CD28 (mAb 9.3). After stimulation, the cells were harvested, washed with PBS, then resuspended and lysed in 1× lysis buffer (Promega, Madison, WI). The

protein concentration of the lysates was determined using the Bio-Rad Protein Assay System. Biotinylated protein markers (New England Biolabs, Beverly, MA) and 75 μg of protein from each sample were separated by SDS-PAGE on a 12% acrylamide gel using 1× Tris-glycine buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS). Proteins were transferred to nitrocellulose using the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell according to the manufacturer's instructions. The filter was stained with Ponceau-S stain (0.2% Ponceau-S and 3% TCA) to confirm the equal loading of proteins. The stain was washed away using TBST buffer (50 mM Tris (pH 7.5), 150 mM NaCl, and 0.1% Tween 20) before preblocking the filter in blocking solution (1× TBS (50 mM Tris (pH 7.5) and 150 mM NaCl), 5% serum, and 5% dry milk) for 1 h at room temperature. p21^{SNFT}-specific antisera (no. 1662) was then added at a 1/3000 dilution and was allowed to incubate at room temperature overnight. The filter was washed three times, for 5 min each time, with TBST (to remove nonspecifically bound Abs) and was incubated for 1 h at room temperature in blocking buffer containing HRP-conjugated secondary Abs (New England Biolabs). After three washes with TBST buffer, the filter was developed using SuperSignal Substrate chemiluminescent reagents (Pierce, Rockford, IL) and exposed to film.

Results

p21^{SNFT} dimerizes with Jun to bind an AP-1 sequence and can functionally repress AP-1 activity

p21^{SNFT} was purified and cloned from a HTLV-I-transformed T cell line by its ability to bind to the HTLV-I LTR *tax*-responsive elements (D. Kolk and W. Wachsman, manuscript in preparation). This novel 127-aa protein contains a bZIP domain and is most closely related to the bZIP transcription factor JDP-1, as determined by amino acid homology. JDP proteins are able to dimerize with Jun family members, but not with Fos proteins, and can bind as a heterodimer with Jun on AP-1 sequences (TREs) (20). To demonstrate that p21^{SNFT} has the same characteristics, a TRE probe was used in an EMSA to analyze DNA binding activity in nuclear extracts from the Jurkat T cell line. As shown in Fig. 1*A*, *lane 1*, there is little binding on the TRE probe when nonstimulated nuclear extracts are used. Upon stimulation with the phorbol ester PMA and the calcium ionophore ionomycin, a lower and a higher migrating complex form (*lane 2*). Excess unlabeled TRE DNA (*lane 3*) competes for binding activity and therefore confirms the specificity of both complexes for the AP-1 sequence. Jun proteins are found in both complexes, because a pan-Jun antiserum cocktail supershifts both species (*lane 4*), while a Fos antiserum cocktail supershifts only the higher migrating complex (*lane 5*). Interestingly, p21^{SNFT} is present only in the lower complex, because only this complex is completely disrupted by the addition of a p21^{SNFT}-specific antisera (*lane 6*). The upper complex is not affected by this antiserum. The effect of the p21^{SNFT} antiserum cannot be explained by its ability to cross-react with Fos or Jun proteins, because the Fos/Jun heterodimer is unaffected by the addition of the antiserum. Therefore, this binding study indicates that p21^{SNFT} binds a consensus TRE with Jun proteins just as related proteins, B-ATF, MEQ, and JDP-1, have been demonstrated to do (20–22). In addition, the complex that contains Jun/Fos heterodimers appears to exclude p21^{SNFT}, and, conversely, the p21^{SNFT}-containing complex excludes Fos.

The binding of p21^{SNFT} in Jun-containing, but not Fos-containing, complexes is also seen using recombinant proteins. Bacterially purified DNA binding and protein dimerization domains of c-Fos and c-Jun were used in combination with purified GST-SNFT and control GST proteins in EMSAs. With the protein concentrations used, neither Fos (Fig. 1*B*, *lane 1*), Jun (*lane 2*), nor p21^{SNFT} (*lane 3*) binds independently to the TRE probe. Fos/Jun heterodimers form when the two proteins are combined (*lane 4*). The addition of 1× (*lane 5*) and 5× (*lane 6*) molar excesses of p21^{SNFT} to Fos and Jun causes the formation of a larger complex in a dose-dependent fashion. This high m.w. complex migrates at the same rate as the

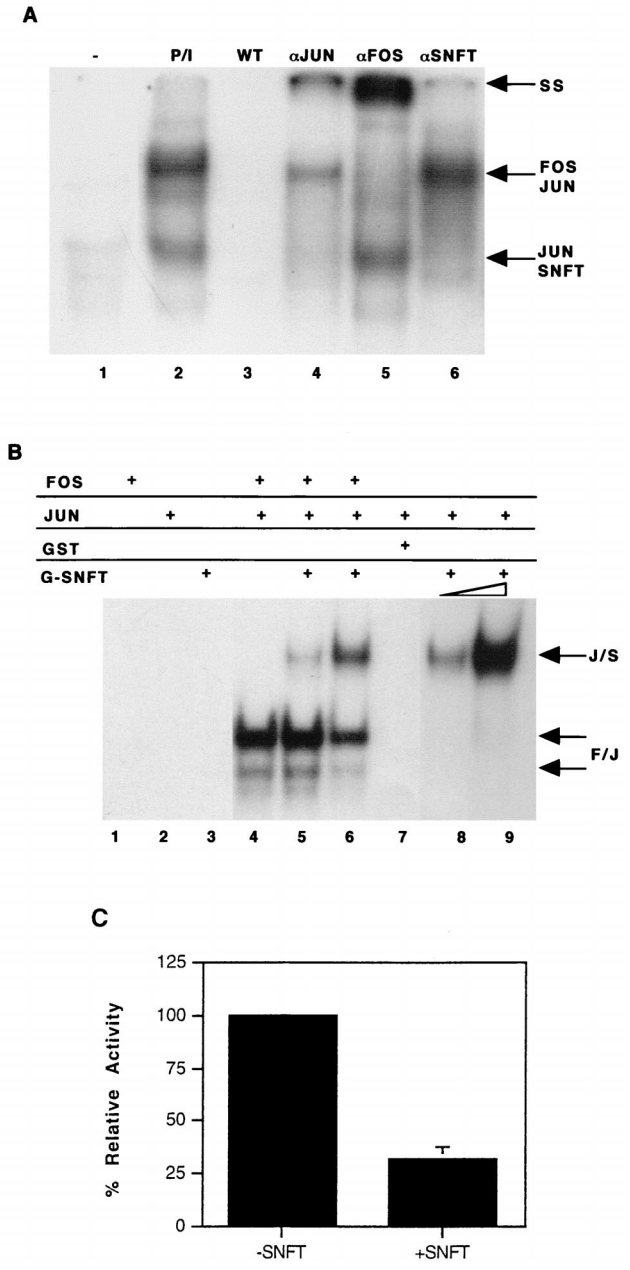


FIGURE 1. p21^{SNFT} binds a TRE sequence with Jun proteins and can inhibit AP-1 activity. *A*, EMSA analysis was performed on a TRE probe using 5 μg of unstimulated (*lane 1*) or PMA/ionomycin-stimulated (*lanes 2–6*) Jurkat nuclear extracts. A 100-fold excess of cold TRE (wild-type (WT)), pan-Jun, pan-Fos, or p21^{SNFT} antiserum was used to determine the specificity and identity of each protein complex. The data are representative of at least three separate experiments. *B*, Binding of purified c-Fos, c-Jun, GST, and GST-SNFT (100 nM of each) to a TRE probe was also analyzed. The proteins added to each sample are indicated above the figure with a +, and the proteins that make up each complex are indicated on the *right margin*. Note that the purified p21^{SNFT} contains the GST tag, resulting in a total size of 47 kDa, while the purified c-Fos was only 32 kDa. Consequently, the Jun/p21^{SNFT} complex is larger than the Fos/Jun complex. This is the opposite of what is seen with the endogenous proteins in nuclear extracts in *A*. *C*, A TRE-controlled reporter construct was transiently cotransfected into Jurkat cells in the presence of the p21^{SNFT} expression construct pCI/SNFT. Transfectants were stimulated with PMA and ionomycin. The data are the average of three controlled experiments.

Jun/p21^{SNFT} complex (*lanes 8* and *9*), indicating the composition of the complex to be Jun and p21^{SNFT} only. It is important to note that the increase in Jun/p21^{SNFT} complexes causes a concomitant decrease in Fos/Jun binding. There is no evidence for a trimolecular complex containing Fos, Jun, and p21^{SNFT}.

The functional consequence of the ability of p21^{SNFT} to interact with Jun on the TRE is the repression of AP-1 enhancer activity (Fig. 1*C*). A reporter gene controlled by multiple TRE enhancers was transiently transfected into Jurkat cells in the presence or the absence of a p21^{SNFT} expression construct (pCI/SNFT). Transfectants were stimulated with PMA and ionomycin, which together mimic TCR stimulation. Fig. 1*C* shows the negative effects of p21^{SNFT} overexpression, where it results in a 68% inhibition of TRE activity compared with that in samples not overexpressing p21^{SNFT}. This reproducible inhibition confirms the original studies of the repressive effects of p21^{SNFT} on AP-1 activity (W. Klump and W. Wachsman, manuscript in preparation).

p21^{SNFT} specifically decreases IL-2 promoter activity in transient transfections

Transient transfections were used to determine whether p21^{SNFT} had an effect on IL-2 promoter activity, because IL-2 relies heavily on the activity of AP-1 for proper activation. Jurkat cells were cotransfected with an IL-2 promoter reporter construct in the presence of increasing amounts of pCI/SNFT. Transfectants were stimulated with PMA/ionomycin. The titration study shows that p21^{SNFT} inhibits IL-2 promoter activity in a dose-dependent fashion (Fig. 2*A*).

Under physiologic conditions, the IL-2 promoter requires simultaneous stimulation of the TCR and CD28 receptors for full activation (27, 28). Signals originating from the TCR activate NF-κB, NF-AT, and AP-1 transcription factors, while CD28 stimulation further increases AP-1 and NF-κB activity and ultimately leads to increased binding on the CD28RE/AP-1 site of the IL-2 promoter (29–32). Therefore, studies were performed to address whether CD28 stimulation could affect the repression seen by p21^{SNFT}. The IL-2 promoter reporter construct was transfected into Jurkat in the presence and the absence of p21^{SNFT} overexpression, as was a reporter construct driven by the CD28RE/AP-1 element from the IL-2 promoter (8, 10). The effect of p21^{SNFT} was analyzed in cells stimulated with PMA/ionomycin in the presence or the absence of CD28 engagement using the stimulating mAb 9.3 (Fig. 2*B*). These data show that p21^{SNFT} represses the CD28RE/AP-1 sequence as well as the IL-2 promoter and that the repressive effects of this protein are not overcome by CD28 stimulation (Fig. 2*B*).

Stimulation does not alter p21^{SNFT} expression

PMA/ionomycin stimulation is known to activate IL-2 gene expression, but it is not known how these stimuli affect p21^{SNFT} protein levels. To understand the expression of endogenous p21^{SNFT} in response to these stimuli, Western analysis was performed on Jurkat whole cell extracts made from cells stimulated for various time periods with PMA/ionomycin as well as cells stimulated with PMA alone or in conjunction with ionomycin and CD28 stimulation for 4 h (Fig. 3). The expression data show that endogenous p21^{SNFT} protein levels do not significantly change at any of the time points or conditions used compared with those in unstimulated cells. Although PMA or PMA/ionomycin stimulation appear to cause a slight decrease in p21^{SNFT} levels at 4 h (compared with values at time zero), CD28 costimulation does not affect p21^{SNFT} levels. Because PMA/ionomycin/CD28 stimulation is optimal for IL-2 production, p21^{SNFT} levels per se do not correlate with IL-2 promoter activity. In addition, p21^{SNFT} protein expression did not alter over the time course and conditions tested

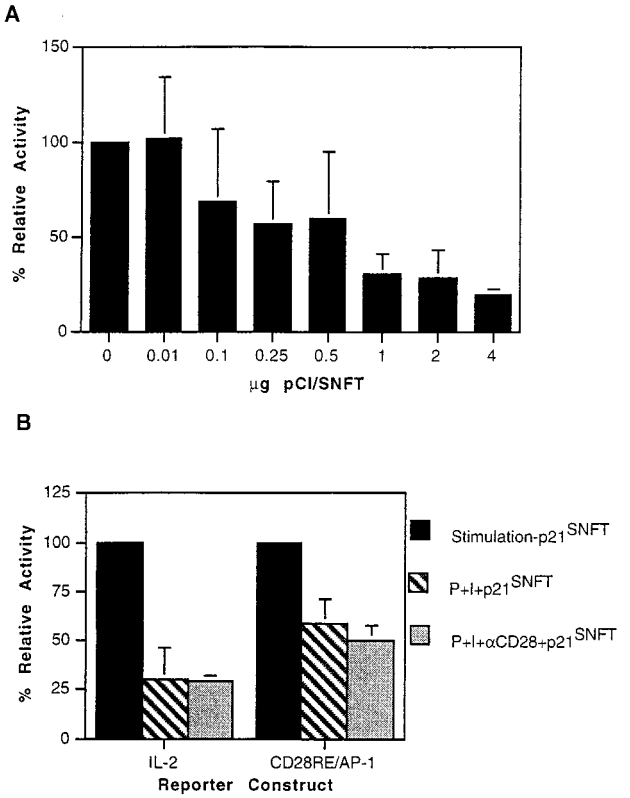


FIGURE 2. p21^{SNFT} inhibits IL-2 promoter activity in Jurkat cells. The activity of the p21^{SNFT}-containing samples is shown as a percentage of the activity observed in the absence of p21^{SNFT}, which was normalized to 100%. The data represent the average of three controlled experiments. *A*, Cotransfections were conducted using the pIL-2-Luc construct in the presence of increasing amounts of pCI/SNFT expression vector. Total DNA concentration remained constant. *B*, pIL-2-Luc and pCD28RE/AP-1-Luc were transfected in the absence (empty vector) or the presence of pCI/SNFT, and the cells were stimulated with PMA/ionomycin with or without anti-CD28. The samples without p21^{SNFT} were set at 100% activity; therefore, the data presented do not reflect the ~3-fold increase in activity seen with P/I/anti-CD28 compared with P/I simulation.

when nuclear extracts were analyzed (data not shown), indicating that nuclear localization of p21^{SNFT} is not affected by stimulation that leads to IL-2 expression.

p21^{SNFT} specifically down-regulates IL-2 promoter activity

The specificity of p21^{SNFT} for the IL-2 promoter was investigated by testing the relative strengths of many viral and human cellular promoters in the presence of overexpressed p21^{SNFT}. Transient transfections of Jurkat cells with a variety of promoter constructs

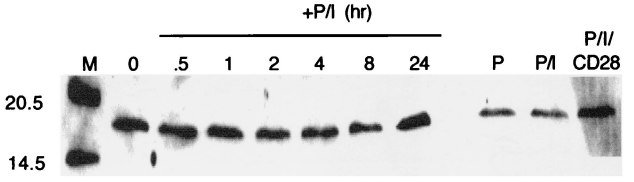


FIGURE 3. p21^{SNFT} is constitutively expressed in Jurkat cells under various stimulation conditions and times. Western analysis of p21^{SNFT} levels was performed using 75 μg of Jurkat whole cell extracts from cells stimulated with PMA/ionomycin (P/I) for various periods or stimulated for 4 h with PMA or PMA/ionomycin/anti-CD28 as indicated. The data are representative of three experiments.

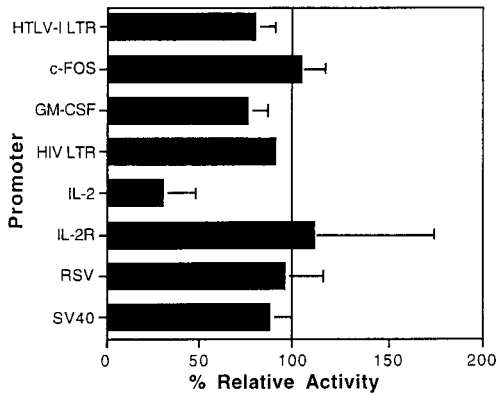


FIGURE 4. p21^{SNFT} specifically inhibits IL-2 promoter activity. CAT (HTLV-I 3′ LTR, c-Fos, −404/+41; HIV 5′ LTR, IL-2, −575/+55; IL-2R, −1240/−227; RSV 3′ LTR, SV40 early promoter) and luciferase (GM-CSF, −620/+37) reporter vectors containing the indicated retroviral and human gene promoters were transiently cotransfected in the absence (empty pCI vector) or the presence of the pCI/SNFT expression vector. The transfectants were stimulated with PMA/ionomycin, and data shown are a percentage of the activity observed in the absence of p21^{SNFT}, which was normalized to 100%. The data are the average of three experiments.

clearly show that of the promoters shown, only the IL-2 promoter is significantly repressed (70% inhibition) by p21^{SNFT} (Fig. 4). This result indicates that p21^{SNFT} acts not as a general transcription inhibitor, but, rather, as a selective inhibitor of IL-2 promoter activity.

Overexpression of p21^{SNFT} leads to repressed endogenous IL-2 gene expression

To verify the biological significance of p21^{SNFT} inhibition of IL-2 promoter activity, the ability of p21^{SNFT} to inhibit expression of the endogenous IL-2 gene was determined. To accomplish this, stable Jurkat T cell lines were generated to constitutively overexpress p21^{SNFT}. Stable oligoclonal lines transfected with either an empty vector (J-CI-1 and J-CI-2) or the p21^{SNFT} expression construct (J-SNFT-1 and J-SNFT-2) were compared for p21^{SNFT} expression. As shown in Fig. 5*A*, the J-CI-1 and -2 control lines express low levels of endogenous p21^{SNFT} protein. In contrast, p21^{SNFT} is markedly increased in the J-SNFT-1 and -2 lines. When IL-2 mRNA levels were analyzed 4 h after PMA and ionomycin stimulation, an average 67% decrease in message was seen in the J-SNFT lines compared with the control J-CI cells (Fig. 5*A*). The percent decrease in IL-2 mRNA levels seen in the J-SNFT lines is equivalent to the repression of IL-2 promoter activity by overexpressed p21^{SNFT} in transient transfection, therefore supporting the hypothesis that p21^{SNFT} is inhibiting the production of IL-2 at the level of transcription initiation.

The p21^{SNFT} overexpressing and control Jurkat lines were also analyzed for IL-2, IL-3, and GM-CSF cytokine expression by ELISA on culture supernatants from stimulated cells. These three cytokines were chosen because all three genes respond to CD28 costimulation through their CD28 response elements (27, 28, 33). Fig. 5*B* shows an average 77% decrease in IL-2 production in PMA/ionomycin-stimulated J-SNFT cells compared with J-CI cells and a 71% decrease with the addition of CD28 stimulation. In contrast, GM-CSF (Fig. 5*B*) and IL-3 (data not shown) were expressed at equivalent levels in the J-CI control and J-SNFT lines, indicating that p21^{SNFT} does not globally inhibit endogenous cytokine production in T cells.

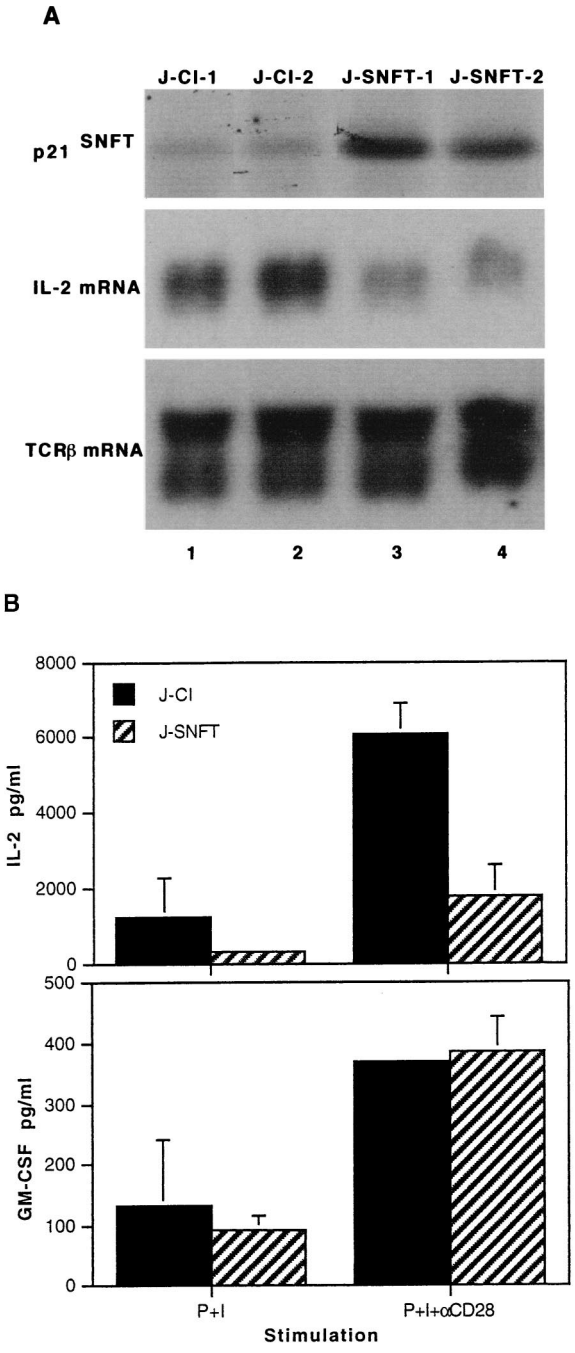
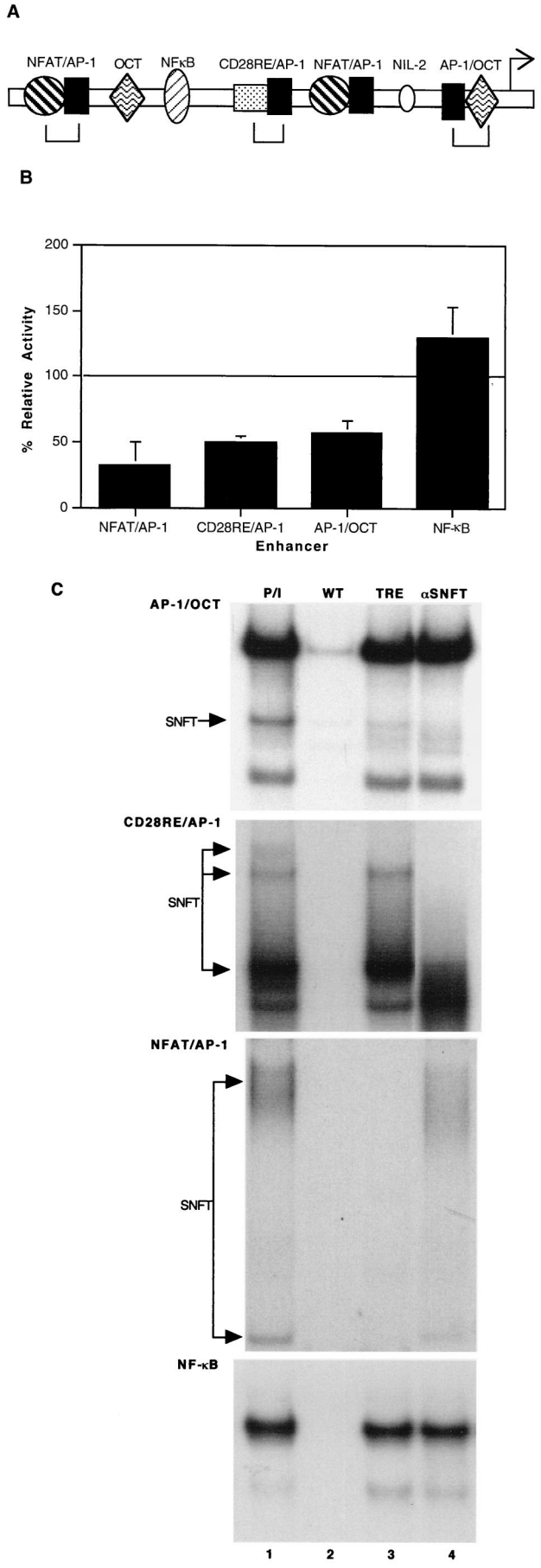


FIGURE 5. Stable Jurkat cell lines overexpressing p21^{SNFT} have decreased IL-2 protein expression. *A*, p21^{SNFT} immunoprecipitations from two PMA/ionomycin (P+I)-stimulated Jurkat control (*lanes 1* and *2*) and two p21^{SNFT}-overexpressing (*lanes 3* and *4*) stable cell lines (*top*). IL-2 mRNA levels (*middle*) were assessed by Northern blot analysis and were compared with TCRβ mRNA expression (*bottom*) as a control for RNA loading. *B*, Culture supernatants from control and p21^{SNFT}-overexpressing lines were assayed for IL-2 and GM-CSF cytokine levels by ELISA after a 4-day stimulation with PMA/ionomycin with or without anti-CD28. The average cytokine production by the control or p21^{SNFT}-overexpressing lines is shown and represents two PMA/ionomycin-stimulated and one PMA/ionomycin/anti-CD28-stimulated experiments.

Multiple AP-1-containing sites within the IL-2 promoter are targets of p21^{SNFT} repression

Because p21^{SNFT} interacts with Jun family members and inhibits the activity of AP-1, p21^{SNFT} may decrease IL-2 promoter activity



by targeting the sites in the IL-2 promoter that require AP-1 binding. This includes the AP-1/OCT, CD28RE/AP-1, and NF-AT/AP-1 elements. A schematic of the IL-2 promoter and the relative positions of these elements is shown in Fig. 6*A*. To determine whether these sites are potential targets of $p21^{SNFT}$ repression, these AP-1-containing elements from the IL-2 promoter were tested for responsiveness to $p21^{SNFT}$ along with a reporter construct containing NF-κB elements. These data, shown in Fig. 6*B*, demonstrate that the AP-1-associated IL-2 promoter elements tested are inhibited by an average of 50% in the presence of $p21^{SNFT}$, as is the TRE element (Fig. 1*B*), while no inhibition of NF-κB was observed. These results suggest that $p21^{SNFT}$ is exerting its effect on the IL-2 promoter through multiple sites, simultaneously inhibiting the activities of a variety of enhancer sites that require AP-1 to function.

$p21^{SNFT}$ is present in protein complexes that it can functionally repress

To determine whether the functional effect of $p21^{SNFT}$ on the IL-2 enhancers is due to a direct or an indirect mechanism of action, protein binding to the IL-2 enhancer elements was analyzed by EMSA. To determine the presence or the absence of $p21^{SNFT}$ in complexes that form on the AP-1/OCT, CD28RE/AP-1, NF-AT/AP-1, and NF-κB sequences, an antiserum specific for $p21^{SNFT}$ was employed. When extracts from PMA/ionomycin-stimulated Jurkat cells are used, protein complexes form on each IL-2 element (Fig. 6*C*, *lane 1*). Competition for protein binding by the addition of a 100-fold excess of unlabeled wild-type probe shows the specificity of these proteins for their respective sequences (*lane 2*). AP-1/OCT (7), CD28RE/AP-1 (9, 10), and NF-AT/AP-1 (12, 34–36) have all been reported to bind AP-1 proteins in cooperation with other transcription factors for their full activity. Therefore, to confirm AP-1 binding activity on each of these IL-2 enhancers, an excess of unlabeled TRE probe was added to out-compete AP-1 binding activity in the nuclear extracts. As shown, the AP-1/OCT, CD28RE/AP-1, and NF-AT/AP-1 probes each possess TRE-specific complexes (*lane 3*). The addition of $p21^{SNFT}$ antiserum to the samples blocked the formation of several, but not all, of the complexes that formed on these three sequences (*lane 4*). Conversely, NF-κB binding remained unaffected by both the TRE competition and addition of $p21^{SNFT}$ antisera.

The ability of the $p21^{SNFT}$ antisera to disrupt complexes found on the OCT/AP-1, CD28RE/AP-1, and NF-AT/AP-1 sequences, but not on the NF-κB sequence, correlates with the ability of $p21^{SNFT}$ to repress the activity of these three enhancers. These data strongly suggest that the mode of action for $p21^{SNFT}$ is direct and

FIGURE 6. $p21^{SNFT}$ can inhibit the activity of AP-1-associated IL-2 promoter enhancer elements and can be found in the protein complexes that form on each sequence. *A*, A diagram of the various enhancer elements found throughout the human IL-2 promoter (−315 to +55). Brackets indicate the sites used in the analysis in *B*. *B*, Reporter constructs containing multimers of isolated IL-2 enhancers or an NF-κB element driving luciferase expression were transfected in the presence or the absence of the pCI/SNFT expression plasmid. The activity of the $p21^{SNFT}$-containing samples is shown as a percentage of the activity observed in the absence of $p21^{SNFT}$, which was normalized to 100%. The data are representative of three controlled experiments. *C*, Probes containing the IL-2 promoter enhancer sequences AP-1/OCT, CD28RE/AP-1, NF-AT/AP-1, and NF-κB were incubated with 5 μg of nuclear extract from PMA/ionomycin-stimulated Jurkat cells. Samples were exposed to a 100-fold excess of unlabeled wild-type (*lane 2*) or TRE competitor (*lane 3*) DNA and $p21^{SNFT}$ (*lane 4*) antisera. Protein complexes that are TRE specific and contain $p21^{SNFT}$ are indicated with arrows. The data are representative of three experiments.

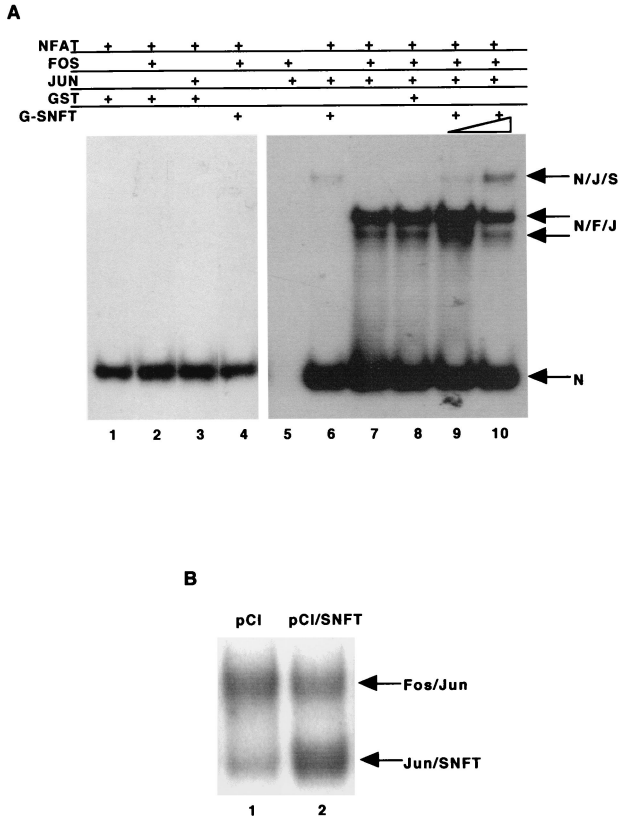


FIGURE 7. p21^{SNFT} binds in complexes with NF-AT and Jun. *A*, Binding of 20 nM bacterially purified NF-ATp and 100 nM c-Fos, c-Jun, GST, and GST-SNFT was analyzed by EMSA on the NF-AT/AP-1 sequence. The proteins added to each sample are indicated above the figure with a +. The proteins that make up each complex are indicated in the *right margin*. Note that the *right side* of the figure is overexposed with respect to NF-AT binding to better visualize the upper complex. *B*, TRE binding was analyzed using control Jurkat (pCI) and p21^{SNFT}-overexpressing (pCI/SNFT) cells to determine whether Fos and p21^{SNFT} can compete for Jun binding in nuclear extracts. Five micrograms of nuclear extract was used in the analysis, and the proteins that make up the two major complexes, as determined with specific antisera (Fig. 1), are indicated in the *right margin*. The data are representative of three experiments.

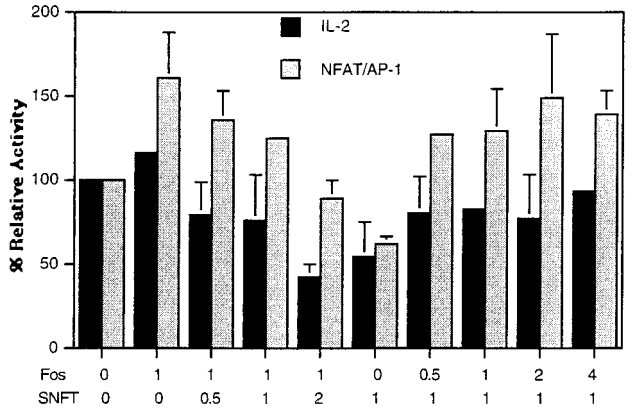


FIGURE 8. p21^{SNFT} and c-Fos overexpression functionally compete with each other to influence IL-2 promoter and NF-AT/AP-1 transcriptional activities. IL-2 and NF-AT/AP-1 reporter constructs were transfected into Jurkat cells with varying amounts of the c-Fos and p21^{SNFT} expression constructs as indicated. The transfectants were stimulated with PMA/ionomycin, and data are expressed as a percentage of the activity observed in the absence of c-Fos or p21^{SNFT}, which was normalized to 100%. The data are the average of two transfections.

probably requires protein-protein interactions with resident Jun proteins, simultaneously affecting the activities of multiple complexes found on the IL-2 promoter.

p21^{SNFT} participates in protein complexes with NF-AT and Jun

For a more detailed analysis of p21^{SNFT}'s participation in DNA binding complexes that include AP-1, additional EMSA analysis of NF-AT/AP-1 binding was conducted using bacterially purified proteins. This site was chosen for further investigation for two reasons: 1) because NF-AT and AP-1 proteins have been found to bind to each of the three enhancers shown to be inhibited by p21^{SNFT} overexpression, and 2) because of knowledge of the constituent proteins that make up the functional complex in vivo (34–36). This information has allowed reconstitution of the complex in vitro, where the protein-protein interactions among NF-AT, Fos, and Jun have been carefully studied (12, 37–39). The major question to be addressed here is whether p21^{SNFT} participates in a complex containing NF-AT, Fos, and Jun or whether it competes with Fos for Jun dimerization, as it appears to do on the consensus TRE (Fig. 1, *A* and *B*). To answer this question, truncated His-tagged NF-ATp, c-Jun, and c-Fos proteins, which contain the DNA binding and protein dimerization domains necessary to form a stable tertiary structure, were used in conjunction with purified GST and full-length GST-SNFT.

Fig. 7 demonstrates the ability of NF-AT to bind to the NF-AT/AP-1 probe (*lane 1*) and the inability of either Fos (*lane 2*) or Jun (*lane 3*) to bind with NF-AT in the presence of the control GST protein. When NF-AT, Fos, and p21^{SNFT} are incubated together, no new complex forms (*lane 4*). This result is expected, because Fos cannot bind with NF-AT to the DNA (*lane 2*), nor can it dimerize with p21^{SNFT}. c-Fos and c-Jun are unable to bind the probe at detectable levels in the absence of NF-AT (*lane 5*), as previously described (12, 34). Conversely, NF-AT, Jun, and p21^{SNFT} can form a complex together, as shown in *lane 6*. NF-AT, Fos, and Jun bind the probe together to form a major (upper) and a minor (lower) complex (*lane 7*). It is unclear what the difference between the two complexes is, because they form only when all three proteins are present, indicating the requirement for the three proteins in the formation of both species. One possible explanation is that the complex can exist in one of two conformations and that these migrate at different rates. Nevertheless, the addition of the control GST protein does not affect these complexes (*lane 8*), but the addition of a 1× (*lane 9*) and a 5× (*lane 10*) molar excess of p21^{SNFT} causes the formation of a new complex. This larger complex comigrates with the NF-AT/Jun/p21^{SNFT} complex, indicating that it also consists of NF-AT, Jun, and p21^{SNFT}. There is no evidence that NF-AT, Fos, Jun, and p21^{SNFT} can exist in a complex together. This observation in addition to a decrease in the NF-AT/Fos/Jun complex as p21^{SNFT} is added strongly suggest that p21^{SNFT} represses NF-AT/AP-1 activity by competing with Fos for Jun binding. Identical results were produced using purified full-length c-Fos and c-Jun proteins (data not shown), eliminating the possibility that the truncated AP-1 proteins lack sequences that are required for the above interactions.

If p21^{SNFT} competes with Fos for Jun binding in vivo as in does in vitro, one would expect to see an increase in p21^{SNFT}/Jun dimer formation relative to Fos/Jun dimers in cells overexpressing p21^{SNFT}. To determine whether this is the case, nuclear extracts from Jurkat control (J-CI-1) and p21^{SNFT}-overexpressing (J-SNFT-1) lines were used to analyze TRE binding activity. The

TRE probe was chosen because of the relatively simple binding pattern, which gives clear resolution of Fos/Jun and $p21^{SNFT}$/Jun dimers (see Fig. 1*A*). Comparison of J-CI-1 (*lane 1*) and J-SNFT-1 (*lane 2*) TRE binding activities shows an increase in the formation of $p21^{SNFT}$/Jun dimers and a decrease in Fos/Jun dimers in the $p21^{SNFT}$-overexpressing line. This is the expected result if Fos and $p21^{SNFT}$ can compete in vivo, thus suggesting a model where an increase in the $p21^{SNFT}$ concentration results in a decrease in Fos/Jun dimer formation.

$p21^{SNFT}$ functionally competes with c-Fos to inhibit transcriptional activity

To functionally test the competition model in vivo, Jurkat cells were transfected with varying amounts of c-Fos and $p21^{SNFT}$ expression vectors. The activities of the IL-2 promoter and the NF-AT/AP-1 sequence were tested under conditions where either c-Fos or $p21^{SNFT}$ expression was held constant while the expression of the other protein was increased. As shown in Fig. 8, increasing the expression of $p21^{SNFT}$ represses IL-2 promoter and NF-AT/AP-1 activities in the presence of c-Fos overexpression in a dose-dependent fashion (*lanes 1–5*). Also, an increase in c-Fos expression rescues the inhibition seen by $p21^{SNFT}$ alone (*lanes 6–10*). The repression by $p21^{SNFT}$ is more pronounced on the NF-AT/AP-1 sequence than on the IL-2 promoter, because c-Fos overexpression alone increases NF-AT/AP-1 activity by 61%, but only 16% on the IL-2 promoter. The discrepancy in the ability of c-Fos to *trans*-activate the two reporter constructs may be because the high endogenous expression of Fos family members that occurs under the stimulation protocol used may be sufficient for maximal induction of the IL-2 promoter but not for the NF-AT/AP-1 sequence. These in vivo studies strongly support a model where $p21^{SNFT}$ inhibits AP-1 and IL-2 promoter transcriptional activity by competing with Fos for Jun binding.

Discussion

The studies presented here demonstrate that $p21^{SNFT}$ represses endogenous IL-2 gene expression via its interaction with several *cis*-acting promoter sites, including the AP-1/OCT, CD28RE/AP-1, and NF-AT/AP-1 elements. $p21^{SNFT}$ directly interacts with these sequences in nuclear extracts either via protein-DNA and/or protein-protein interactions with other transcription factors that have specificity for the DNA. The strong correlation between the down-regulation of Jun-responsive elements and the presence of $p21^{SNFT}$ in these complexes suggests that a requirement for the actions of $p21^{SNFT}$ lies with a critical interaction with Jun proteins.

The EMSAs preformed with purified proteins on both the TRE and NF-AT/AP-1 sequences suggest that $p21^{SNFT}$ inhibits AP-1 and NF-AT/AP-1 activities by competing with Fos for Jun dimerization. $p21^{SNFT}$ is most likely accomplishing this via leucine zipper domain interactions with Jun. The evidence for this is that GST-SNFT can physically interact with truncated c-Jun protein even though it lacks 186 of its N-terminal amino acids. This truncation eliminates the *trans* r-activation domain, but retains the basic leucine zipper domain. c-Jun does not bind with NF-AT in the EMSAs in the absence of c-Fos or $p21^{SNFT}$, but the addition of either protein causes the cooperative binding of NF-AT and Jun to the DNA. The ability of AP-1 to cooperatively bind DNA with NF-AT is a well-documented observation (12, 37), but the data presented suggest that this interaction is not limited to AP-1, because Jun/$p21^{SNFT}$ dimers also have this function.

The model of competitive inhibition by $p21^{SNFT}$ has been previously proposed, but not experimentally tested, for the repressive effects of B-ATF and JDP-2 on AP-1 activity (20, 21). This model would not require $p21^{SNFT}$ protein levels to change, because regulation would occur at the level of Fos expression. Indeed, the constant level of endogenous $p21^{SNFT}$ protein present under various stimulation conditions may be sufficient to out-compete for Jun binding when the levels of Fos are very low, such as in unstimulated or suboptimally stimulated T cells (40). The lack of Fos in Jun/$p21^{SNFT}$ complexes results in decreased transcriptional activity, most likely due to the absence of activation domains within Fos that cooperatively work with Jun to induce transcription (41–44). In situations where $p21^{SNFT}$ levels are in excess of Fos levels, a tighter level of control could be achieved in repressing AP-1 enhancers when optimal conditions are not met for AP-1 activity. Conversely, when T cells are stimulated via the TCR and the CD28 receptor, they transcriptionally up-regulate the c-*fos* gene through activation of the Ras/Raf/MEKK/MEK pathway (40). A transiently high level of Fos protein quickly ensues, which can compete with and overcome the level of $p21^{SNFT}$ for Jun dimerization. This dynamic between Fos and $p21^{SNFT}$ is reflected in the titration experiment, where excess c-Fos was shown to eliminate the repressive effects of $p21^{SNFT}$ in vivo.

The inhibition of IL-2 promoter activity by $p21^{SNFT}$ is intriguing, in that no other promoter tested was similarly affected. Although it is shown that three AP-1-containing enhancer elements within the IL-2 promoter are targets of the repression, it is important to note that some of the other promoters tested in this analysis, including SV40 (45, 46) and GM-CSF (47), also contain functional AP-1 binding sites, yet their activities remain unaffected by $p21^{SNFT}$. One explanation for this result may be due to the different context of *cis*-acting elements upstream or downstream of the TRE(s) contained within these promoters. This may be particularly true in the case of GM-CSF, because the gene contains an enhancer with three NF-AT/AP-1 sites located ~3 kb upstream of the gene and one at −54 (48, 49), yet neither endogenous GM-CSF levels nor the activity of the promoter tested is significantly affected by overexpression of $p21^{SNFT}$. The SV40 promoter contains only two AP-1 sites, whereas the IL-2 promoter has been reported to contain at least four functional AP-1 sites (50).

A competitive mode of action by $p21^{SNFT}$ may be complicated by additional levels of control such as post-transcriptional modifications. It is important to note that many bZIP transcription factors are functionally regulated by phosphorylation. The phosphorylation status of bZIP factors such as Fos, Jun, and CREB/ATF is controlled by the upstream kinases FRK, JNK, and PKA, respectively (40, 51). The activities of these nuclear proteins require phosphorylation at specific sites, and it is therefore possible that $p21^{SNFT}$ is also regulated in such a fashion. The fact that $p21^{SNFT}$ protein levels are relatively constant under a variety of stimulation protocols leaves open the possibility that it may be regulated by other means. Finally, the exact role that $p21^{SNFT}$ has in the processes that control IL-2 production requires further investigation, but potential areas of inquiry are where IL-2 production is limited, such as in T cell anergy or T cell differentiation.

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