Regulation of NF-κB, AP-1, NFAT, and STAT1 Nuclear Import in T Lymphocytes by Noninvasive Delivery of Peptide Carrying the Nuclear Localization Sequence of NF-κB p50

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Regulation of NF-κB, AP-1, NFAT, and STAT1 Nuclear Import in T Lymphocytes by Noninvasive Delivery of Peptide Carrying the Nuclear Localization Sequence of NF-κB p50

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Activation of T lymphocytes by Ags or cytokines results in translocation of the transcription factors NF-κB, AP-1, NFAT, and STAT from the cytoplasm into the nucleus. The first step in the nuclear import process is recognition of a nuclear localization sequence (NLS) within the karyophilic protein by a cytoplasmic receptor such as the importin (karyopherin)-α subunit. The NLSs of NF-κB, AP-1, and NFAT differ and the NLS of STAT1 has not yet been identified. Herein we demonstrate that the inducible nuclear import of NF-κB, AP-1, NFAT, and STAT1 in Jurkat T lymphocytes is significantly inhibited by a cell-permeable peptide carrying the NLS of the NF-κB p50 subunit. NLS peptide-mediated disruption of the nuclear import of these transcription factors results in inhibition of IkBα and IL-2 gene expression, processes dependent on NF-κB or the combination of NF-κB, AP-1, and NFAT. Furthermore, we show that inhibitory NLS peptide interacts in vitro with a cytoplasmic NLS receptor complex comprised of the Rch1/importin (karyopherin)-β heterodimer expressed in Jurkat T cells. Taken together, these data indicate that the inducible nuclear import of NF-κB, AP-1, NFAT, and STAT1 in Jurkat T cells can be regulated by NLS peptide delivered noninvasively to the cytoplasm of Jurkat T cells to target members of the importin (karyopherin)-αβ NLS receptor complex. The Journal of Immunology, 1998, 161: 6084–6092.
pore and into the nucleus by an energy-dependent process involving the GTPase Ran/TC4 and the Ran interacting factor NTF2/p10 (22). Homologues of importin-α (60 kDa) have been described in several species including Xenopus, yeast, Drosophila, mouse, and human. To date, in addition to Rch1/hSRP1α (33, 34) and Npl-1/hsRPR1 (35, 36), four other human importin-α homologues have been cloned including Qip1 and hSRP1γ (37–40). Importin-β (karyophilin-β1, β2) (97 kDa) has also been characterized in several different species (41–43). The second pathway of nuclear import involves an importin-β-related protein, termed transportin or karyophilin-β3, which binds the M9 sequence present in the hNRP A1 protein, but does not interact with classical or bipartite NLSs (43, 44). Initial studies have shown that transportin or its yeast homologue, Kap104p, can perform both the NLS-binding and nuclear pore docking functions of the heterodimeric importin-αβ complex (44, 45).

To investigate the mechanism by which the nuclear import machinery recognizes NF-κB, AP-1, NFAT, and STAT1, the new strategy of noninvasive peptide delivery was used (46). Using this technique, peptides bearing the NF-κB p50 NLS were delivered into the cytoplasm of intact cells and the activation-dependent nuclear import of each transcription factor analyzed in the presence of the NLS peptides. Our results demonstrate that the NF-κB p50 NLS peptide significantly inhibited the signal-dependent nuclear import of NF-κB, AP-1, NFAT, and STAT1 and the subsequent induction of genes activated by one or more of these factors in human T lymphocytes. We also show that the inhibitory p50 NLS peptide binds in vitro to the NLS receptor complex, Rch1/importin-β. These findings indicate that despite the presence of diverse NLS motifs in these transcription factors, the nuclear import of NF-κB, AP-1, NFAT, and STAT1 can be regulated by a single NLS peptide delivered noninvasively to the cytoplasm of human T cells to target members of the importin (karyophilin) family of NLS receptors (e.g., Rch1/importin-β). This inhibition of transcription factor nuclear import was subsequently coupled to attenuation of gene transcription.

### Materials and Methods

#### Reagents

The peptides listed in Table I were synthesized manually by a stepwise solid-phase synthesis method using Boc chemistry as previously described (46). Polycyclonal antiserum specific for IκBα (amino acids 1–29) and NF-κB p50 (amino acids 1–21) were generated as described (12). Polyclonal c-Jun antiserum and affinity-purified polyclonal c-Fos Ab were gifts from Dr. Stephen Hann (Vanderbilt University, Nashville, TN). The mAb 7A6, specific for human NFATc (NFAT2), was a gift from Dr. Gerald Crabtree (47). Polyclonal Abs and mAbs to ISGF3/STAT1 (Transduction Laboratories, Lexington, KY) were gifts from Dr. Mark Boothby (Vanderbilt University). Polyclonal Abs to Rch1 and importin-β were gifts from Dr. Dirk Görlich (41). Affinity-purified horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG were purchased from Pierce (Rockford, IL). Expression plasmids encoding full-length human Rch1 and importin-β were generously provided by Dr. Dirk Görlich (41). Reombinant Rch1 and importin-β were expressed in Escherichia coli and purified from cell lysates by standard chromatographic procedures using Q Sepharose Fast Flow followed by Superdex 200 (Pharmacia Biotech, Piscataway, NJ).

#### Peptide treatment and cell extract preparation

Human Jurkat T cells were grown in RPMI 1640 (Cellgro, Fisher, Atlanta, GA) supplemented; with 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% heat-inactivated FBS. For each assay, 5 × 10⁶ Jurkat T cells were pelleted, suspended in 4.5 ml fresh culture media, and incubated at 37°C (5% CO₂) for 60 min. Peptides were dissolved in 0.5 ml HBSS without Ca²⁺ or Mg²⁺ (Cellgro) and added to the cells for 30 min before the addition of either a combination of PMA (5 μM; Sigma, St. Louis, MO) or ionomycin (2 μM; Calbiochem, La Jolla, CA) or IFN-γ (1 U/ml; Sigma). Nuclear extracts prepared from IFN-γ-stimulated cells were made more concentrated than extracts prepared from cells stimulated with PMA/ ionomycin because of the relatively lower concentration of STAT1 found in Jurkat T cells. Samples were vortexed for 15 min at 4°C, centrifuged, and the supernatant saved as the nuclear extract. The protein concentration of the nuclear extracts was determined using the Pierce BCA protein assay and equalized among samples using buffer B. For cells treated with IFN-γ, buffers A and B also contained 1 mM Na₃VO₄ and 10 mM NaF. Intracellular peptide concentrations were determined by cell extract ELISA. Briefly, cytoplasmic and nuclear extracts from peptide-treated cells were combined and 25, 50, or 100 μl used to coat the wells of a 96-well plate. For comparison, adjacent wells were coated with extracts from untreated cells to which known amounts of peptide were added. Following a 16-h incubation, all wells were washed with PBS plus 0.05% Tween 20, blocked with BSA, and incubated with an Abs specific for the LMP residues present in each peptide (see Table I) (46). Peptide-Ab complexes were detected with anti-rabbit IgG conjugated with alkaline phosphatase and quantitated by ELISA.

#### Electrophoretic mobility shift assay (EMS)

The following oligonucleotide sequences, with the transcription factor recognition sites underlined, were used: NF-κB, 5'-AGGTAGAGGAGGACATTCCCGAGAGGA-3' (8); AP-1, 5'-GATCTTTACATTGGAAAATTTTAT-3'; NFAT, 5'-AGCTTAGAGGGGACTTTC-3' (49); NFAT-AP-1, 5'-GATCCAGAAGGGAAAAACTGGTTCTACAG-3' (18); STAT1, 5'-GATCTGTCAGACATTCCGTAAATC-3' (51); and NF-Y, 5'-GATCTGAGATTCTGTGAGTCCTTGGGAGCTTGG-3' (52).

Double-stranded oligonucleotides (40 pmol) were labeled with 50 μCi [α³²P]dATP (3000 Ci/mmol, DuPont-New England Nuclear, Boston, MA) and 5 U of E. coli DNA polymerase I (Klenow fragment) at room temperature for 30 min. Labeled oligonucleotides were separated from unincorporated [α³²P]dATP by chromatography on Sephadex G50 (Sigma). DNA-binding reactions were performed in a final volume of 20 μl of NF-κB- and AP-1-binding reactions contained 5 μl nuclear extract, 1 μg poly dI-dC, 1 μg salmon sperm DNA, EMSA buffer (20 mM HEPES pH 7.9, 5 mM glucose, and 1 mM EDTA), and 100,000 cpm [³²P]labeled oligonucleotide. NFAT- and STAT1-binding reactions contained 5 μl nuclear extract, 2 μg poly dI-dC, and 100,000 cpm [³²P]labeled oligonucleotide. NF-Y-binding reactions contained 5 μl nuclear extract, 2 μg poly dI-dC, EMSA buffer, and 50,000 cpm [³²P]labeled oligonucleotide. All binding reactions were incubated at room temperature for 20 min and terminated by the addition of 2 μl of 10X EMSA gel-loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, and 50% glycerol), 5 μl of NF-κB- and AP-1-binding reactions were run in 4% polyacrylamide/0.5X TBE (45 μm Tris-borate and 1 mM EDTA) gels prerun in 0.5X TBE for 60 min at 100V. STAT1 samples were run in 4% acrylamide/Tris-glycine (50 mM Tris, 380 mM glycine, and 2 mM EDTA) gels as described previously (8). Gels were dried onto chromatography paper (Whatman XChr; VWR, Atlanta, GA) and exposed to Fuji BAS-IIIs phosphorimager plates (Fuj, Tokyo, Japan) for quantitation and then to Kodak Biomax MR autoradiography film (Eastman Kodak, Rochester, NY). Ab supershift analyses of DNA-binding complexes were

### Table I. Sequences of cell-permeable and non-cell-permeable peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Cell-Permeable Sequence</th>
<th>– – –NLS– – –</th>
</tr>
</thead>
<tbody>
<tr>
<td>N50</td>
<td>VQRRDKLMP</td>
<td></td>
</tr>
<tr>
<td>SN50</td>
<td>AAVALLPAVALLAELPVQRRDKLMP</td>
<td></td>
</tr>
<tr>
<td>SN50M</td>
<td>AAVALLPAVALLAELPVQRRQKLMP</td>
<td></td>
</tr>
<tr>
<td>SM</td>
<td>AAVALLPAVALLAELPVQARDQKLMP</td>
<td></td>
</tr>
<tr>
<td>CYN50</td>
<td>CVYRRDKLMP</td>
<td></td>
</tr>
<tr>
<td>CYN50M</td>
<td>CVYRRKGGKLMP</td>
<td></td>
</tr>
</tbody>
</table>

"Sequences are given in single letter amino acid code with bold type indicating basic residues important for NLS function. Underlined residues constitute the cell-permeable sequence derived from the hydrophobic (b) domain of the Kaposi fibrolasts growth factor signal sequence (46)."
performed by adding subunit-specific antisera to the nuclear extract immediately before addition of other binding-reaction components. EMSAs to detect AP-1 in cytoplasmic extracts were performed as described above with the exception that the nuclear extract was replaced with 10 μl of 10x concentrated cytoplasmic extract prepared by centrifugation in Microcon-30 concentrators (Amicon, Beverly, MA).

**Immunoblotting**

Nuclear proteins (50 μg) were precipitated with 10% TCA and the protein pellet resuspended in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2 mM EDTA, 10% glycerol, 2% SDS, 2% 2-ME, and 0.025% bromphenol blue). Samples were heated at 100°C for 5 min and run in a 10% SDS polyacrylamide mini gel (53). Cytoplasmic proteins (100 μg) were mixed with SDS-PAGE sample buffer, heated at 100°C for 5 min and run in an 8% SDS polyacrylamide mini gel (53). Proteins were transferred to nitrocellulose (Pharmacia Biotech) and blocked with 5% dry milk in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) at room temperature for 1 h. Immunoreactive proteins were detected using Abs specific for NF-κB p50 (1:8000), c-Jun (1:6000), NFATc (1:6000), IκBα (1:2500), Rch1 (1:5000), or importin-β (1:5000). After incubation with primary Abs, blots were incubated with goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated Ab (1:20,000; Pierce), developed with supersignal horseradish peroxidase enhanced chemiluminescence reagent (Pierce), and exposed to Kodak Biomax MR film.

**RNA isolation and ribonuclease protection assays**

Total RNA was prepared from 10⁷ Jurkat T cells using Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s recommendations. Anti-sense RNA probes were generated using the Riboprobe Gemini system (Promega, Madison, WI) according to the manufacturer’s recommendations. The human IL-2 anti-sense RNA probe (280 nucleotides) was generated from pGEMIL-2 (a gift from Drs. Geraldine Miller and Marty Reich, Vanderbilt University). The human β-actin anti-sense RNA probe (255 nucleotides) was generated from a plasmid provided by Boehringer-Mannheim (Mannheim, Germany). Ribonuclease protection assays were performed using the RPA II system (Ambion, Austin, TX) as described by the manufacturer. Briefly, 30 μg Jurkat total RNA and 160,000 cpm ³²P-labeled IL-2 probe or 10 μg Jurkat total RNA and 80,000 cpm ³²P-labeled β-actin probe were coprecipitated with ethanol. Pellets were resuspended in hybridization buffer, heated to 90°C for 3 min, and annealed at 42°C for 16 h. Annealed samples were digested with RNase A and T₁, and precipitated with ethanol. The resulting precipitate was resuspended in gel-loading solution, heated to 90°C for 3 min, and run in a 5% polyacrylamide/TBE gel containing 8 M urea. The gel was exposed to Kodak Biomax MR film and then to Fuji BAS-IIIs phosphorimager screens for quantitation.

**Preparation of NLS peptide/biotinylated-BSA conjugates and in vitro binding assay**

The cysteine-modified NF-κB p50 wild-type (CYN50) and mutant (CYN50 M) NLS peptides (Table I), were conjugated to BSA and the peptide-BSA conjugates were biotinylated as described (54). The NLS peptide/biotinylated-BSA conjugate (20 μg) and BSA (200 μg) were added to 100 μl of Jurkat T cell whole-cell extract and incubated at 4°C for 60 min with gentle rocking. Aggregates were removed by centrifugation in a microcentrifuge at 10,000 rpm for 10 min (4°C). The supernatant was incubated at 4°C for 60 min with 25 μl of packed ultralink immobilized streptavidin (Pierce). Following incubation, bound proteins were washed six times with wash buffer (20 mM Tris-Cl, pH 7.5, 80 mM NaCl, and 2 mM MgCl₂) and eluted with 200 μl of 1 M MgCl₂. Eluted proteins were precipitated with 10% TCA, run on an 8% SDS-PAGE mini gel, and Western blotted with Abs to Rch1 and importin-β.

**Results**

**Kinetics of the signal-dependent nuclear import of NF-κB, AP-1, NFAT, and STAT1**

Jurkat T cells were stimulated with either a combination of PMA and ionomycin or IFN-γ. The combination of PMA and ionomycin mimics costimulation through the TCR and CD28 (55). The level of transcription factor nuclear import was determined by measuring DNA-binding activity using an EMSA performed on nuclear extracts. As shown in Fig. 1, the DNA-binding activities of NF-κB, AP-1, NFAT, and STAT1 were maximally induced by 30, 60, 10, and 10 min, respectively. The relatively slower rate of induction of AP-1 DNA-binding activity in nuclear extracts is consistent with the requirement for de novo AP-1 synthesis following agonist stimulation. Because NFAT DNA binding to a human DNA probe requires AP-1, the kinetics of NFAT induction are coincident with that of AP-1. However, NFAT binding to a murine DNA probe (50), independent of AP-1 complexation, showed a rapid kinetics. During the same experimental time period, the constitutively expressed nuclear factor, NF-Y, as indicated. NS underneath STAT1 in the lower panel indicates a nonspecific, constitutive band.

**Inhibition of transcription factor nuclear import by a cell-permeable NLS peptide**

We have previously shown that a cell-permeable peptide carrying the NLS from NF-κB p50 (SN50, Table I) blocks the nuclear import of NF-κB in endothelial and monocytic cell lines (46). Because AP-1 and NFAT contain NLSs that differ from the p50 NLS,

![FIGURE 1. Kinetics of the signal-dependent nuclear import of NF-κB, AP-1, NFAT, and STAT1.](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC5248988/bin/fig1.png)
and the STAT1 NLS has not yet been identified, we used the SN50 peptide to test whether the signal-dependent nuclear import of these transcription factors can be regulated by the same NLS peptide that blocked NF-κB nuclear translocation. As shown in Fig. 2, the SN50 peptide inhibited not only NF-κB nuclear import but also the nuclear import of AP-1, NFAT, and STAT1. In respect to NFAT, inhibition was observed toward both its forms, one requiring AP-1 for DNA binding and detectable with human DNA probe (NFAT/AP-1 in Fig. 2), and a second form that does not require AP-1 complexation for binding to murine DNA probe (NFAT in Fig. 2). The inhibitory activity of the SN50 peptide was concentration-dependent (Fig. 2) and was evident for up to 2 h of stimulation (data not shown). Within the time frame of this experiment, the constitutively expressed NF-Y was not affected by the SN50 peptide. Nevertheless, a possibility of reduction of NF-Y content in the nuclear extract by longer treatment of cells with SN50 peptide cannot be excluded.

We found that the ability of the SN50 peptide to inhibit STAT1 nuclear import depends on the concentration of IFN-γ used to stimulate the cells. As shown in Fig. 3, the SN50 peptide (75 μM) efficiently inhibited STAT1 nuclear import in cells stimulated with a low dose of IFN-γ (1 U/ml), but this inhibitory effect was not observed in cells stimulated with a high dose of IFN-γ (100 U/ml). In this experiment, it is apparent that the levels of STAT1 DNA-binding activity in nuclear extracts from cells stimulated with low or high doses of IFN-γ were similar (data not shown). Together, these data indicate that the loss in efficacy of the SN50 peptide at high doses of IFN-γ is not caused by an increased cytoplasmic pool of activated STAT1 saturating the peptide inhibition of the import process.

Because stretches of basic amino acids in nucleic acid-binding proteins sometimes serve as both the NLS and the DNA- or RNA-binding domain (56), we next tested whether the SN50 peptide interfered directly with in vitro DNA-binding reactions (EMSA). We found that the DNA-binding activity of NF-κB, AP-1, NFAT, and STAT1 was not affected when SN50 peptide (20 μM final concentration) was added to EMSA reactions (data not shown).

Nuclear import inhibition by the SN50 peptide requires the NLS motif

To determine whether the observed inhibition of NF-κB, AP-1, NFAT, and STAT1 nuclear import by the SN50 peptide was dependent on the p50 NLS motif, we assessed the inhibitory effect of cell-permeable peptides containing mutations in the NLS sequence. Fig. 4A shows that the inhibitory effect of the SN50 peptide on the nuclear import of all four transcription factors was significantly reduced when only 2 of 10 NLS residues were mutated (SN50M). The inhibitory effect was completely lost when 7 of 10 NLS residues were mutated (SM). To confirm the EMSA results, Western blots were performed on the same nuclear extracts. As shown in Fig. 4B, SN50 peptide caused a significant reduction in the intranuclear levels of NF-κB p50, c-Jun, and...
NFATc proteins. The mutant peptide, SN50M, was much less effective in blocking transcription factor nuclear import. However, some reduction in the intranuclear levels of these proteins, in particular NFATc, was evident in cells treated with the SN50M peptide and this result was consistent with the reduction in the DNA-binding activities observed by EMSA (Fig. 4A). In these studies, the SM peptide did not significantly affect the intranuclear levels of any of these transcription factors. As expected, no inhibitory effect was seen with the p50 NLS peptide lacking the cell-permeable hydrophobic sequence (N50) necessary for cellular delivery of the peptides (46).

In consideration of the results described above, it was necessary to assess whether there were variations in the cellular uptake of the wild-type and mutant NLS peptides. Therefore, we determined the relative intracellular peptide concentrations in extracts of cells treated with the same concentration of 75 μM using quantitative ELISA. Using a polyclonal IgG that recognized the LMP sequence present at the C terminus of each peptide (see Table I) (46), this assay demonstrated that both cell-permeable peptides reached an intracellular level corresponding to approximately 4% of the total peptide added to the cells, whereas the non-cell-permeable N50 peptide was not detected (data not shown).

The appearance of AP-1 in the nucleus following cellular activation requires de novo synthesis of c-Fos and c-Jun (57). Therefore, it is possible that the inhibitory effect of the SN50 peptide on AP-1 nuclear import might be due to inhibition of the nuclear import of other factors necessary for expression of these genes. We tested this possibility by measuring the level of AP-1 in cytoplasmic extracts of peptide-treated and control cells after PMA/ionomycin stimulation. As determined by EMSAs of concentrated cytoplasmic extracts, cells stimulated for 60 min in the presence of a polyclonal IgG that recognized the LMP sequence present at the C terminus of each peptide (see Table I) (46), this assay demonstrated that both cell-permeable peptides reached an intracellular level corresponding to approximately 4% of the total peptide added to the cells, whereas the non-cell-permeable N50 peptide was not detected (data not shown).

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FIGURE 4. Nuclear import inhibition by SN50 requires the NLS motif. A, Jurkat T cells were treated with the peptides indicated above the lanes (see Table I), each at a final concentration of 75 μM, for 30 min and then stimulated with 5 nM PMA/2 μM ionomycin for 60 min or 1 U/ml IFN-γ for 15 min. Nuclear extracts were prepared and assayed for transcription factor DNA-binding activity by EMSA as described in Materials and Methods. The figure shows only the sections of autoradiograms that included mobility-shifted complexes containing specific 32P-labeled DNA and NF-κB, AP-1, NFAT, STAT1, or the constitutively expressed nuclear factor, NF-Y, as indicated. NS underneath STAT1 in the lower panel indicates a nonspecific, constitutive band. B, Western blot analysis of the levels of NF-κB p50 subunit, AP-1 c-Jun subunit, and NFATc in nuclear extracts from peptide-treated cells. Extracts used in this analysis were the same as those used for EMSA in A, and immunoblotting was done as described in Materials and Methods. C, Cytoplasmic extracts prepared from cells stimulated as in A and assayed for AP-1 transcription factor DNA-binding activity by EMSA as described in Material and Methods. Note that in SN50-treated cells there is a partial reduction in AP-1 DNA-binding complexes present in the cytoplasm, whereas there is near complete inhibition of AP-1 nuclear import as judged by the lack of DNA-binding complexes in the nucleus (A).
the SN50 peptide contained approximately 60% AP-1 present in the cytoplasm of control cells (Fig. 4C). In contrast, SN50 caused near complete (>97%) inhibition of AP-1 nuclear import as compared with control cells (Fig. 4A). These results indicate that SN50 exerted a twofold effect on AP-1: First, by decreasing the amount of c-Fos and c-Jun synthesized and second, by inhibiting the nuclear import of subunits that are synthesized.

Inhibition of transcription factor nuclear import blocks IκBα and IL-2 gene expression

Because the induction of IκBα gene transcription is dependent primarily on NF-κB (58–61), we determined whether the SN50 peptide blocked IκBα expression. Fig. 5A shows that, in the absence of the SN50 peptide, IκBα was rapidly degraded within 20 min after PMA/ionomycin stimulation and that the steady-state levels of IκBα were reestablished after 90 min due to its rapid de novo synthesis. To evaluate the effect of the SN50 peptide on the normal process of IκBα degradation and resynthesis, we prepared cytoplasmic extracts from peptide-treated and control cells 20 and 90 min after stimulation with PMA/ionomycin. As shown in Fig. 5B, IκBα degradation proceeded normally in the presence of SN50 peptide and the other peptides used in this study, indicating that they did not interfere with extracellular signal-induced phosphorylation and proteolysis of IκBα. However, 90 min after stimulation a concentration-dependent inhibitory effect of the SN50 peptide on IκBα resynthesis was observed, while neither the SM nor the N50 control peptide significantly inhibited IκBα resynthesis. Thus, inhibition of nuclear import of NF-κB by SN50 peptide resulted in concomitant inhibition of IκBα gene expression.

The induction of IL-2 gene expression depends on NF-κB, AP-1, and NFAT (62). Therefore, we investigated whether blockade of the nuclear import of these transcription factors by the SN50 peptide exerted an inhibitory effect on IL-2 gene transcription. First, we analyzed the kinetics of IL-2 mRNA induction in Jurkat T cells in the absence of peptide. Using a quantitative ribonuclease protection assay, we found that IL-2 mRNA reached easily detectable levels after 90 min of stimulation with PMA/ionomycin (data not shown). Consequently, we determined the effect of the SN50 peptide on IL-2 mRNA synthesis 90 min after stimulation. As shown in Fig. 6, the SN50 peptide caused a concentration-dependent inhibition of inducible IL-2 mRNA synthesis, but did not inhibit the constitutive synthesis of β-actin mRNA. At the concentration of 75 μM used in many experiments, the inhibition of IL-2 mRNA synthesis by SN50 peptide was 52 ± 7.5% (mean ± SEM).

Interaction of inhibitory SN50 peptide with cytoplasmic NLS receptor complexes

Our next objective was to determine whether inhibitory NLS peptide interacts with the known cytoplasmic NLS receptor complexes expressed in Jurkat T cells. As shown in Fig. 7A, Rch1 and importin-β were detected in Jurkat T cell extracts. In these experiments, purified recombinant Rch1 and importin-β were included as positive controls for Ab reactivity. To determine whether the Rch1/importin-β complex in Jurkat T cells was capable of binding to the NLS sequence of NF-κB p50 NLS peptide, in vitro binding experiments were performed using either the p50 wild-type (CYN50) or mutant (CYN50 M) NLS peptides (see Table I) conjugated to biotinylated BSA. To capture NLS receptor complexes, the NLS peptide/biotinylated-BSA conjugates were incubated with whole-cell extracts from Jurkat T cells. The bound proteins were

FIGURE 5. Inhibition of IκBα synthesis by the cell-permeable SN50 peptide. A, A section of a Western blot that demonstrates the kinetics of degradation and resynthesis of IκBα in Jurkat T cells that were stimulated with 5 nM PMA/2 μM ionomycin for the times indicated. Cytoplasmic extracts were prepared and assayed by immunoblotting as described in Materials and Methods. B, A section of a Western blot that demonstrates the specific inhibition of IκBα resynthesis by the SN50 peptide following agonist-induced degradation. Jurkat T cells were treated with the indicated peptides (see Table I) for 30 min at the concentrations shown, and then stimulated with 5 nM PMA/2 μM ionomycin for 20 min or 90 min as indicated. Extract preparation and analysis was the same as in A.

FIGURE 6. Inhibition of IL-2 mRNA synthesis by the cell-permeable SN50 peptide. Shown is an autoradiogram of a polyacrylamide gel used to analyze 32P-labeled anti-sense RNA fragments protected from RNase A and T1 digestion by IL-2 or β-actin mRNA. Jurkat T cells were treated with SN50 peptide (Table I) for 30 min at the concentrations indicated and then stimulated with 5 nM PMA/2 μM ionomycin for 90 min. Total RNA was prepared and the levels of IL-2 and β-actin transcripts were analyzed using a ribonuclease protection assay as described in Materials and Methods.
recovered using streptavidin beads and analyzed by Western blotting. As shown in Fig. 7B, Rch1 and importin-β interacted with the wild-type NLS peptide/biotinylated-BSA conjugate. The reduction of Rch1 and importin-β binding to the mutant peptide/biotinylated-BSA conjugate indicates the specificity of this interaction. However, there was residual binding of Rch1 and importin-β to the mutant peptide. This residual binding provides an explanation for the observation that the nuclear import of transcription factors was still partially inhibited by the mutant peptide in vivo (see Fig. 4). As shown in Fig. 7C the specificity of Rch1 and importin-β binding to the wild-type NLS peptide/biotinylated-BSA conjugate was further supported by the demonstration that this interaction was inhibited by a 50-fold molar excess of the SN50 peptide used in experiments performed in intact Jurkat T cells. These results indicate that the inhibitory SN50 peptide targets the Rch1/importin-β NLS receptor complex present in Jurkat T cell extracts.

Discussion

It is well-recognized that signaling to the nucleus plays a key role in the response of T lymphocytes to immune and inflammatory stimuli. The steps involved in this signaling include: 1) antigenic peptide-dependent activation of the TCR/CD3 complex or cytokine-dependent activation of cognate receptors; 2) signal transduction mediated by cytoplasmic kinases and phosphatases; 3) mobilization of various transcription factors, including NF-κB, NFAT, and STAT, which are sequestered in the cytoplasm, or AP-1 following its de novo synthesis; 4) import of transcription factors into the nucleus where they bind to DNA and activate transcription of a large subset of genes; and 5) nucleocytoplasmic export of some transcription factors, e.g., NFAT. Because distinct signal transduction pathways are involved in the mobilization of NF-κB, AP-1, NFAT, and STAT1 to the nucleus following agonist stimulation, it was important to determine whether a common nuclear import pathway(s) involved in signaling by each of these transcription factors can be regulated by a single NLS peptide derived from the NF-κB p50 and delivered noninvasively to the cytoplasm of Jurkat T cells. The results presented here indicate that following stimulation of Jurkat T cells with PMA/ionomycin or low doses of IFN-γ the nuclear import of NF-κB, AP-1, NFAT, and STAT1 is inhibited by SN50 peptide. The same SN50 peptide inhibited the stimulus-dependent nuclear import of NF-κB in endothelial and monocytic cell lines (46). The SN50 peptide containing NLS made of basic amino acids is unlikely to interfere with the nuclear export of NFAT mediated by Ran and CRM1 (63) and enhanced by glycogen synthase kinase-3 (64).

These data expand the range of inhibition of nuclear import by SN50 peptide to three other transcription factors important for T cell function in the immune system. Unlike the NF-κB and AP-1 proteins that require classical or bipartite NLSs for nuclear import, the NFATp/c proteins can be efficiently targeted to the nucleus by a short sequence having only three basic residues (KRR or KRK) located in the amino-terminal segment of the protein (30, 31). CALCINEURIN-mediated dephosphorylation of nearby serine residues is postulated to expose this region for binding by the nuclear import machinery (30). The sequences required for activation-dependent nuclear import of STAT1 have not yet been identified but inspection of the STAT1 amino acid sequence (65) reveals three clusters of basic amino acid residues that have been postulated to function as NLSs. However, recent deletion and mutagenesis experiments have shown that none of these sequences serve as the NLS of STAT1, and it has been suggested that a unique type of NLS is present within this protein (32).

We found that the SN50 peptide efficiently inhibited the nuclear import of STAT1 in Jurkat T cells stimulated with low doses of IFN-γ, but with high doses this inhibitory effect was significantly decreased. In contrast, we have determined that SN50 peptide inhibition of NF-κB, AP-1, and NFAT nuclear import does not change even when a 20-fold higher concentration of agonist PMA, in combination with ionomycin, is used to stimulate the cells (data not shown). Because the levels of activated STAT1 in both nuclear

**FIGURE 7.** Expression of the nuclear import proteins Rch1 and importin-β in Jurkat T cells and in vitro binding to NLS peptides. **A,** Western blot analysis of Rch1 and importin-β protein expression in Jurkat T cells. Blots were treated with Abs to Rch1 or importin-β as indicated above each blot. Lanes are labeled to indicate the source of protein as either the specified recombinant protein or cell extract from Jurkat T cells. Whole cell extracts and recombinant proteins were prepared and assayed by immunoblotting as described in Materials and Methods. The molecular masses of protein standards (Life Technologies) are shown in kDa. **B,** Binding of Rch1 and importin-β to wild-type and mutant NF-κB NLS peptides. Whole cell extracts from Jurkat T cells were incubated with wild-type or mutant p50 NLS peptides (Table I) that were conjugated to biotinylated BSA. Proteins bound to the NLS peptide/biotinylated-BSA conjugate were recovered using streptavidin beads, and immunoblot analysis was performed as described in Materials and Methods. **C,** Inhibition by SN50 peptide of Rch1/importin-β binding to the wild-type NLS peptide conjugated to biotinylated BSA. Western blot analysis shows that 50-fold molar excess of free (unconjugated) SN50 peptide inhibited the binding of the Rch1/importin-β complex to the homologous NLS peptide conjugated to biotinylated BSA.
and cytoplasmic extracts of Jurkat T cells are not significantly different at low and high doses of IFN-γ, the insensitivity of STAT1 nuclear import to inhibition by the p50 NLS peptide at high doses of this cytokine does not appear to be simply a consequence of increased cytoplasmic pools of activated STAT1 overcoming peptide inhibition of the import process. We have also observed in THP-1 monocyteic cells expressing much higher concentration of STAT1 that at both low and high doses of IFN-γ tyrosine phosphorylation of STAT1 occurs normally and is unaffected by the presence of the SN50 peptide, thereby demonstrating that SN50’s effect on STAT1 is not at the level of signal transduction (T.R.T. and J.H., unpublished observations). These findings suggest the intriguing possibility that there are biochemical differences in the mechanism of STAT1 nuclear import that depend on the concentration of IFN-γ and that an alternative import pathway, resistant to inhibition by a classical NLS peptide, is used at higher concentrations. In other cell types (HeLa and 293T), high concentrations of IFN-γ (≥2000 U/ml) induced nuclear import of STAT1 mediated by NPI-1 (hSRP1). Moreover, NLS peptides derived from wild-type SV40 T Ag did not inhibit interaction between NPI-1 and STAT1 (32). These results support our findings that high doses of IFN-γ induce nuclear import of STAT1 that is insensitive to inhibition by NLS peptide. The involvement of transportin (karyopherin-β2) in this alternative pathway seems unlikely because there are no regions within STAT1 with homology to the M9 sequence of hnRNP A1 and transportin pathway is not sensitive to inhibition by peptides representing classical NLS sequences (43, 44, 65). Nevertheless, nuclear import of STAT1 requires IFN-γ-induced phosphorylation of tyrosine 701, dimerization of the subunits, and the GTPase activity of Ran/TC4 (66). The latter is needed for translocation of karyophilic proteins across the nuclear pore following their recognition and docking by either importin-α (karyopherin-αβ1) or transportin (karyopherin-β2) NLS receptors (43).

We show that an inhibitory SN50 peptide containing the NLS of NF-κB p50 (SN50) binds to the Rch1 (importin-α/importin-β) heterodimer. This interaction is specific for the NLS because mutation of two basic amino acid residues within the sequence significantly reduces binding of the peptide to Rch1 and importin-β. These data suggest that the mechanism by which the p50 NLS peptide inhibits nuclear import of NF-κB, AP-1, NFAT, and STAT1 involves direct disruption or blockade of the importin-αβ-transcription factor interaction. Because Rch1 contains the NLS-binding domain (67), it is likely that the p50 NLS peptide binds directly to this subunit of the importin-αβ complex, an interpretation supported by the observation that the SN50 peptide (see Table I) binds recombinant Rch1 but not importin-β in vitro (data not shown). The interaction of NLS peptides derived from different karyophilic proteins in human T lymphocytes varies in respect to different importin-α (karyopherin-α) proteins (68). It is plausible that more than one importin-α (karyopherin-α) protein interacts with transcription factors analyzed in these experiments.

The inhibition of the nuclear import of more than one transcription factors by SN50 peptide as reported herein has practical implications in view of growing use of this reagent as an inhibitor of the NF-κB nuclear translocation (69, 70). Interpretation of these data should take into account the expanded range of SN50 peptide inhibitory activity toward nuclear import of transcription factors other than NF-κB. The SN50 peptide also inhibited inducible expression of the IκBα and IL-2 genes, processes dependent on NF-κB alone or the combination of NF-κB, AP-1, and NFAT (58–62). In contrast to the IκBα (MAD3) gene regulated primarily by NF-κB, expression of IL-2 requires complex synergy between NF-κB, AP-1, and NFAT and can proceed when relatively low concentrations of these factors along with constitutively nuclear factors are attained in the nucleus. Thus, partial inhibition of nuclear import of NF-κB, AP-1, and NFAT by the SN50 peptide at 50 μM was apparently insufficient to cause significant inhibition of IL-2 mRNA expression. Higher concentrations of the SN50 peptide (75–100 μM) are required to achieve ≥50% inhibition of IL-2 mRNA expression at 90 min following stimulation of Jurkat T cells. This is, to our knowledge, the first evidence that gene expression can be regulated in intact cells by the inhibition of transcription factor nuclear import at the level of NLS recognition. These findings are significant because they offer a new strategy to exert an immunosuppressive effect on intact, activated T cells through blocking nuclear import of key transcription factors involved in T cell immune function.

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