The Serum Response Factor Nuclear Localization Signal: General Implications for Cyclic AMP-Dependent Protein Kinase Activity in Control of Nuclear Translocation

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We have identified a basic sequence in the N-terminal region of the 67-kDa serum response factor (p67SRF or SRF) responsible for its nuclear localization. A peptide containing this nuclear localization signal (NLS) translocates rabbit immunoglobulin G (IgG) into the nucleus as efficiently as a peptide encoding the simian virus 40 NLS. This effect is abolished by substituting any two of the four basic residues in this NLS. Overexpression of a modified form of SRF in which these basic residues have been mutated confirms the absolute requirement for this sequence, and not the other basic amino acid sequences adjacent to it, in the nuclear localization of SRF. Since this NLS is in close proximity to potential phosphorylation sites for the cAMP-dependent protein kinase (A-kinase), we further investigated if A-kinase plays a role in the nuclear location of SRF. The nuclear transport of SRF proteins requires basal A-kinase activity, since inhibition of A-kinase by using either the specific inhibitor peptide PKIm or type II regulatory subunits (RII) completely prevents the nuclear localization of plasmid-expressed tagged SRF or an SRF-NLS-IgG conjugate. Direct phosphorylation of SRF by A-kinase can be discounted in this effect, since mutation of the putative phosphorylation sites in either the NLS peptide or the encoded full-length SRF protein had no effect on nuclear transport of the mutants. Finally, in support of an implication of A-kinase-dependent phosphorylation in a more general mechanism affecting nuclear import, we show that the nuclear transport of a simian virus 40-NLS-conjugated IgG or purified cyclin A protein is also blocked by inhibition of A-kinase, even though neither contains any potential sites for phosphorylation by A-kinase or can be phosphorylated by A-kinase in vitro.

The 67-kDa serum response factor (p67SRF or SRF) is a ubiquitous nuclear transcription factor that acts through binding to a consensus DNA sequence, the serum response element (SRE) (38, 41, 53) present in the promoters of genes involved in the early mitogenic response (15, 52). All functional SREs so far examined contain the core sequence CC(A/T)6GG (termed the CArG sequence), which is also found in the promoters of several muscle-specific genes (cardiac and skeletal actin genes and the dystrophin gene, for example) (22, 34). Through its binding to these different promoters, SRF has been implicated in control of both proliferation and muscle differentiation, which could be confirmed through in vivo immunodepletion with anti-SRF antibody injection (11, 13, 56). Correlating with its transcriptional activity, SRF is a nuclear phosphoprotein present throughout the cell cycle in all cell lines tested to date (13).

Studies of the requirements for nuclear translocation have implicated basic sequences (referred to as nuclear localization signals [NLS]) sufficient to induce nuclear import (5, 9). NLS are generally short stretches of 5 to 10 amino acids containing several basic residues (arginine and/or lysine) (6). Some proteins (e.g., the simian virus 40 [SV40] large-T antigen) have an NLS comprising a contiguous run of basic amino acids (21, 25), while others (e.g., nucleoplasm) have a bipartite motif (7, 10). It is believed that the basic region facilitates interaction with NLS binding proteins (NBPs), which allow transport through nuclear pore complexes in an ATP-dependent manner (1, 47, 50, 59). These NBPs have been characterized for various organisms, and two of them, NBP70 and Nopp140, are phosphorylated in vivo, an event required for their interaction with NLS (31, 51). Moreover, the possibility that nuclear localization might be regulated by variations in specific kinase activities is illustrated by various examples (29, 37, 42). In particular, it has been shown that the SRE binding activity of the transcription factor NFIL-6 is inducible by forskolin treatment, an event which correlates with an increase in both NFIL-6 phosphorylation and its nuclear localization (33). Positive correlations have also been made between phosphorylation and nuclear translocation for c-fos and the cyclic AMP (cAMP)-dependent protein kinase (A-kinase) (35), and suggested for c-fos and A-kinase (45). Recently, we observed that the active nuclear import of the myogenic transcription factor MyoD requires A-kinase activity in a manner that did not involve direct phosphorylation of MyoD (55). This observation raised the following two possibilities for a potential role of A-kinase in this process: (i) phosphorylation by A-kinase of a component distinct from MyoD but specifically involved in MyoD nuclear import is required, and (ii) A-kinase activity is needed in a more general mechanism of control of nuclear transport.

In the present study, we show that SRF contains a unique and highly efficient NLS (SRF-NLS) located at the N-terminal part of the protein. Since this NLS is located in close proximity...
to potential consensus phosphorylation sites for A-kinase, we questioned whether direct phosphorylation by A-kinase could modulate the efficiency of the process of nuclear import. Whereas A-kinase activity was required for NSF nuclear import, site-specific mutations of NSF showed that A-kinase does not exert its control through direct phosphorylation of the sites present near NSF-NLS. Additionally, we show that the nuclear transport of cytoplasmically injected SV40-NLS-conjugated IgGs or purified cyclin A is impaired through inhibition of A-kinase activity. Since neither SV40-NLS nor cyclin A can be phosphorylated by A-kinase, these data show the general requirement for A-kinase in the mechanism of nuclear translocation control.

MATERIALS AND METHODS

Cells. Human foreskin fibroblasts (Hs-68) or rat embryo fibroblasts (REF-52) (26) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 8% fetal calf serum as described previously (13). Cells were plated 2 to 3 days before use.

Peptide synthesis and conjugation. Solid-phase synthesis of the different peptides was performed using a 9050S synthesizer with Fmoc chemistry. PEG-PS resin (Milligen, St-Quentin les Yvelines, France), 9-fluorenyl methyl oxycarbonyl (FMOC) as temporary amino group protection, and benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium-hexafluorophosphate (BOP) as the coupling reagent. Terminal cysteine was added to each peptide sequence for the purpose of protein coupling. Peptides (see sequences below) were purified by reverse-phase high-pressure liquid chromatography on Nucleosil 5C18 columns by using a linear gradient of acetonitrile (0 to 30% gradient) with 0.1% aqueous trifluoroacetic acid. Purified peptides or SRF-D6 (13) was coupled to rabbit immunoglobulin G (IgG) (Sigma, St-Quentin Fallavier, France) by using the water-soluble cross-linking reagent sulfo-succinimidyl-4-[['maleimidomethyl(cyclohexane)-1-carboxylate (Sulfo-SMCC) (Pierce, Interchim, Montlucon, France) and 50:1 molar ratio described by the manufacturer. Conjugates were gel filtered on PD10 (Pharmacia) equilibrated in 100 mM HEPES (N-2-hydroxyethylpiperazin-N-2-ethanesulfonic acid) (pH, 7.2) and resuspended at 2 mg/ml in 100 mM HEPES for subsequent microinjection. The coupling ratio was evaluated to be ca. 2 to 5 peptides per IgG molecule by the shift in molecular weight of the IgG heavy chains by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Sequences of the peptides were as follows (mutated residues are underlined): SRF-NLS,9 [G[A,E,R,G,K]RSLE[104]; SRF-NLS-E7-E100,9 [LGAERRGLKRALAE104]; SRF-NLSAla,9 [G[A,E,R,G,K]RALAE104]; SRF-NLSAsp,9 [G[A,E,R,G,K]RALDE104]; SRF-NLSArg,9 [G[A,E,R,G,K]RDELK104].

In vitro phosphorylation. SRF proteins obtained by in vitro translation in reticulocyte lysate (the plasmid was kindly provided by R. Treisman, Imperial Cancer Research Fund, London, England) were incubated with either casein kinase II (CKII) (11) or A-kinase (Promega, Coger, Paris, France) in the presence of 100 mM [γ-32P]ATP (100 to 200 cpm/pmol) for 20 min at room temperature. The reaction was stopped by adding added buffer to the reaction mixture and then boiling for 2 min before loading the sample onto an SDS–12.5% polyacrylamide gel and performing autoradiography.

Protein kinase A activation in living cells. Cells were made quiescent by removal of serum for 36 h and then incubated with forskolin (15 μM) and MIX (0.5 mM) or 8-Br-cAMP (1 mM) and MIX. At different times after treatment, cells were fixed in 3.7% formalin in PBS for 5 min. After extraction in acetone (–20°C) for 30 s and rehydration in PBS containing 0.2% bovine serum albumin, cells were stained for NSF distribution as previously described (13) and for actin distribution by using rhodamine-phalloidin (8). The stainings were analyzed with a Leica confocal laser scanning microscope.

For nuclear transport studies, a solution of peptide-rabbit IgG conjugates (1 mg/ml) mixed 1/3 with unconjugated mouse IgG (1 mg/ml; Sigma) was injected into the cytoplasm of cells. In some cases, either PKIn (5.10−5 M), PKI (25-4) (5.10−5 M) (8), the regulatory subunit RI of A-kinase (1.4 μg/ml), or the catalytic subunit of A-kinase (0.1 to 1 mg/ml) was included in the injection solution. Cells were fixed at different times after injection and stained for the localization of the NLS-conjugated IgG by using biotinylated anti-rabbit antibody (1:200; Amersham) for 30 min and streptavidin-Texas red (1:400; Amersham) for 30 min and for localization of the mouse IgGs, used as markers for the injected cells, by using fluorescein-conjugated anti-mouse antibody (1:200; Cappel–Organon Teknika, Fresnes, France) for 30 min. The chromatin was stained with Hoechst stain (0.1 μg/ml; Sigma), applied just before cells were mounted and observed by confocal laser scanning microscopy.

Plasmids were injected into the nuclei of proliferating cells by using a solution of plasmid (0.1 mg/ml) containing rabbit marker IgGs (1 mg/ml; Sigma) either alone or together with PKIn. Five hours later, cells were fixed and stained with the plasmid conjugate. The expression of NLS was detected by using fluorescein-conjugated anti-rabbit antibody (1:200; Cappel) and biotinylated anti-tubulin antibody (1:200; Amersham) for 30 min and then with fluorescein-conjugated anti-mouse antibody (1:200; Cappel). Injected cells were stained with fluorescein-conjugated anti-rabbit antibody (1:200; Amersham) for 30 min and then with streptavidin-Texas red (1:400; Amersham).

Solutions of purified human cyclin A containing either mouse marker IgGs or mouse IgGs and PKIn were microinjected in the cytoplasm of Hs-68 cells about 5 to 8 h after serum refeeding (i.e., during the early G1 phase of the cell cycle). After 60 or 1 h, cells were fixed and analyzed for cyclin A distribution as described elsewhere (17).

Confocal laser scanning microscopy. Dual-channel confocal laser scanning microscopy was performed by using the Leica confocal laser scanning microscope equipped with a krypton-argon ion laser with two major emission lines at 488 nm for fluorescein isothiocyanate excitation and 568 nm for rhodamine or Texas red excitation. Planapochromat lenses (40× or 63×) were used, and the untreated images were directly transferred from the VME bus of a Leica Motorola 68040 to a Silicon Graphics, Inc. (SGI, Mountain View, Calif.) IRIS Indigo workstation (IRIS 3000). Images were deconvoluted, gamma mapped, and converted to SGI raster format by using Convert software (18). Figures were assembled completely with SGI Showcase 2.01 and printed directly as postscript files by using a Kodak Colorese thermal sublimation printer.

RESULTS

A basic sequence in the N-terminal part of SRF functions as NLS. Analysis of the primary amino acid sequence of p67SRF reveals four clusters of basic amino acids which could act as potential NLS. From the N terminus of the protein, the first sequence, termed NLS1, is located within the predicted DNA-binding domain. The three other potential NLS (NLS2, NLS3, and NLS4) are located within the predicted DNA-binding domain (Fig. 1a).

The activity of these NLS was assessed by monitoring their capacity to translocate nonnuclear proteins, for example, immunoglobulins (rabbit IgG), into the nucleus following micro-injection into cultured mammalian cells. Rabbit IgG was chosen because of their large size, which effectively prohibits passive diffusion through the nuclear membrane, and their inert nature once injected into cells. The efficiency of NLS1 was tested with the peptide 9 [G[A,E,R,G,K]RSLE[104]. The efficiencies of NLS2, NLS3, and NLS4 were tested simultaneously by using a portion of SRF spanning from residues 113 to 265 and containing the DNA-binding domain of the protein (called SRF-DB) (22). This peptide includes the three other potential NLS (Fig. 1a). Both the NLS1-containing peptide and purified SRF-DB were chemically coupled to rabbit IgG (as detailed in Materials and Methods) and microinjected into the cytoplasm of REF-52 or Hs-68 fibroblasts together with an
FIG. 1. Identification of potential NLS in the amino acid sequence of SRF protein. (a) We have identified the presence of four potential NLS in the primary amino acid sequence of SRF on the basis of the presence of clusters of basic residues (arginine and lysine). NLS were numbered from the N terminus of the protein, with NLS1 being outside the DNA-binding domain and the three others, NLS2, NLS3, and NLS4, being within the DNA-binding domain. For each of these basic sequences, a consensus A-kinase site (R/K-X-S/T or R/K-X-X-S/T) is found associated. SRF-DB (delimited by the horizontal arrow) represents a portion of SRF we have expressed in bacteria that contains NLS2, -3, and -4 and comprises the DNA-binding region of SRF. (b) Either NLS1-containing peptide or purified SRF-DB was coupled to rabbit IgG as described in Materials and Methods. Each of these conjugates was microinjected into the cytoplasm of growing Hs-68 cells with an inert mouse marker antibody for subsequent identification of injected cells. Cells were fixed at different times afterward and stained for the conjugated rabbit IgG and for the mouse marker antibody. Panels A to D show injection of NLS1-IgG conjugates (A and C) fixed at 2 h (B) or 24 h (D), and panels E to H show injection of SRF-DB-IgG conjugates (E and G) fixed at 2 h (F) or 24 h (H). Bar = 10 μM. (c) Growing Hs-68 cells were microinjected with SRF-NLS-IgG conjugates together with mouse marker antibody, fixed at different times afterward, and stained for mouse marker antibody (A, C, and E) and localization of SRF-NLS-IgG conjugates (B, D, and F). Cells were fixed 15 min (A and B), 30 min (C and D), or 60 min (E and F) after injection. Bar = 10 μM.
inert mouse antibody for subsequent identification of injected cells. The localizations of conjugated NLS1, conjugated SRF-DB, and unconjugated rabbit IgGs were analyzed by immunofluorescence at different times thereafter. As shown in Fig. 1b, when Hs-68 cells were injected with conjugated-NLS1-containing peptide (panels A and C), a strong nuclear staining was observed 2 h after injection (panel B) and remained visible after 24 h (panel D). This shows that NLS1 (designated SRF-NLS) is competent to translocate coupled IgGs into the nucleus. As few as two SRF-NLS peptides conjugated per IgG molecule would successfully result in IgG nuclear translocation. In contrast, only cytoplasmic staining was observed in cells injected with IgG-conjugated SRF-DB (Fig. 1b, panels E and G) 2 h (panel F) and 24 h (panel H) after injection, clearly showing that SRF-DB does not contain a sequence efficient in inducing nuclear localization (even at a coupling ratio of two SRF-DB peptides per IgG molecule). Similar kinetic of nuclear import were also observed with IgGs conjugated to the NLS sequence of SV40 (CPKKRKV), a sequence described as being highly efficient in promoting nuclear localization (20).

Taken together, these results show that amongst the four potential NLS present in SRF, only the first N-terminal sequence, NLS1 (henceforth designated SRF-NLS), was able to confer rapid nuclear localization on large and nonnuclear proteins such as IgGs.

Mutation of the basic residues within the NLS sequence of SRF completely prevents its nuclear localization. In order to assess if the NLS sequence we have identified in SRF protein was absolutely required for SRF nuclear transport and to discount the possibility that other sequences in the entire SRF

FIG. 2. Mutation of arginine 95 and arginine 96 of SRF prevents the nuclear import of the expressed tagged protein. Either wild-type (wt) HAP/SRF plasmid (A and B) or mutated HAP/SRF-E95K96 plasmid (C and D), together with a rabbit marker antibody for subsequent identification of injected cells, was injected into the nuclei of growing Hs-68 cells. Five hours later, cells were fixed and stained for rabbit marker antibody (A and C) or for HAP/SLF expression and localization (B and D). Bar = 10 μM.
protein could substitute for it, we examined the effect on SRF nuclear transport of mutating SRF-NLS. To distinguish the expression of the cloned cDNA from the endogenous SRF, we fused a 9-residue influenza virus hemagglutinin peptide epitope (HAP) (YDVPDYASL) to the 5’ end of the SRF cDNA. Expression of the fusion protein (HAP/SRF) was detected with monoclonal antibody 12CA5 (58).

Mutant SRF protein in which the two residues arginines 95 and 96 were substituted with glutamic acid was prepared as described in Materials and Methods. Either wild-type or mutated SRF plasmids (0.1 mg/ml), in a eucaryote expression vector under the regulation of an SV40 promoter, were injected into the nuclei (to effect maximal expression) of growing Hs-68 or REF-52 cells. After 5 h (a time sufficient to allow protein expression), cells were fixed and analyzed for HAP/ SR expression and localization by immunofluorescence. As shown in Fig. 2, when cells were injected with plasmids expressing wild-type SRF (panel A), the expressed epitope-tagged SRF protein was exclusively localized in the nucleus (panel B). No cytoplasmic staining was ever detected, even if expression was monitored for shorter periods of time (data not shown), showing that wild-type SRF is efficiently translocated into the nucleus at all times. In marked contrast, when cells were injected with plasmids encoding SRF mutants in which the two basic residues (arginines 95 and 96) were exchanged for glutamic acid (Fig. 2C), the expressed SRF was exclusively cytoplasmic (panel D), showing that the mutated HAP/SRF fusion protein is no longer competent to translocate into the nucleus. Furthermore, using SRF-NLS peptides in which either arginines 95 and 96 and/or lysine 99 and arginine 100 were substituted with glutamic acid, we have observed that the nuclear localization activity of SRF-NLS peptide required all these basic amino acids (data not shown).

These results confirm the efficiency of the NLS sequence we have identified in SRF for the nuclear transport of the protein and particularly show that this NLS is the essential and unique motif for SRF nuclear localization, with no other sequence in SRF protein substituting for its activity.

Modulation of SRF distribution in a manner dependent on A-kinase activity. The SRF-NLS sequence is located between two phosphorylation sites that appear to be phosphorylated in vivo (19): a potential site for CKII (**SESGEEEEE** and a putative consensus site for either A-kinase, calcium calmodulin (CaM)-dependent kinase II, or the ribosomal S6 kinase p90**rk** (**KRSLS**)). Whereas it has been shown already that SRF is phosphorylated in vitro by p90**rk** (43), we have also observed in vitro phosphorylation of SRF by A-kinase (data not shown). The close proximity of these phosphorylation sites to the NLS and our previous observations of MyoD nuclear localization (55) prompted us to investigate potential involvement of A-kinase-dependent phosphorylation of these sites in the subcellular localization of SRF.

We have previously described the nuclear localization of SRF throughout the cell cycle in mammalian cells (13). In order to monitor the effects of A-kinase activation, we treated cells with forskolin and MIX and subsequently examined the distribution of SRF by confocal microscopy using two fibroblast lines (REF-52 and Hs-68). Since we have previously observed that the maximum effects of A-kinase activation occur in quiescent cells (14), SRF staining was performed with quiescent cells. In order to control for the level of A-kinase activation, we monitored the extent of actin filament reorganization (24), as shown in Fig. 3A and C. In addition, we have carried out measurements of intracellular cAMP concentrations, to compare the cAMP levels in quiescent, serum-stimulated, and forskolin-treated cells. The results of these measurements are summarized in Table 1. Quiescent cells, where the level of cAMP and therefore of active A-kinase is minimal (Table 1), present an intact actin microfilament network (Fig. 3A) and SRF is essentially distributed in the nucleus (Fig. 3B). A low level of cytoplasmic staining is also detectable in these cells because of the high degree of sensitivity of the confocal laser scanning microscope technique (Fig. 3B). When cells were treated for 20 min with forskolin (15 μM) and MIX (0.5 mM), which elevated intracellular cAMP levels by three- to fourfold (Table 1), we observed the complete disappearance of this diffuse cytoplasmic distribution of SRF, concomitant with a more granulated nuclear staining (Fig. 3D). No effects were observed when MIX alone was used (data not shown), which correlates with an absence of detectable changes in cAMP level with MIX alone. The changes shown in Fig. 3A to D are fully representative of the staining observed in all cells treated to activate A-kinase. Similar results for SRF distribution were obtained when 8-Br-cAMP (1 mM) (a cell-soluble analogue of cAMP) was used to activate A-kinase or following microinjection of the highly purified catalytic subunit of A-kinase (data not shown). A 20-min drug treatment was sufficient to allow a strong activation of A-kinase (as shown in Fig. 3C) without inducing extensive morphological modifications that could have contributed to a modification in the SRF distribution. Previous articles have reported that a reduction of the cAMP level occurs before induction of oocyte meiotic division (2, 27, 32) and mitotic entry in mammalian cells (23). We stained cells for the distribution of SRF at the onset of mitosis (i.e., prophase). Figure 3E and F shows an area of growing cells with a cell in prophase in the center (marked by an arrow) discernible by the condensed chromatin in the Hoechst-stained DNA (panel E). In this prophase cell, where the level of A-kinase is low, cytoplasmic SRF staining is clearly visible, whereas none of the surrounding interphase cells show cytoplasmic staining for SRF (Fig. 3F). The appearance of cytoplasmic staining for SRF in prophase cells occurs prior to any sign of nuclear envelope disassembly, as checked by staining cells for nuclear lamina organization (data not shown), and therefore is unlikely to be due to an increased porosity of the nucleus. In addition, we have shown before that throughout the different phases of mitosis, the majority of SRF remains preferentially associated with the condensed chromatin (13). These data illustrate that the subcellular localization of SRF is apparently modulated with respect to variations in intracellular A-kinase activity. The presence of cytoplasmic SRF is detected only when A-kinase levels are reduced during the cell cycle.

We have previously described the effective inhibition of A-kinase activity in vivo, using a modified stable version of the specific inhibitor peptide for A-kinase, PKIm (8). We therefore examined if complete inhibition of intracellular A-kinase activity through microinjection of PKIm modifies the nuclear localization of overexpressed tagged SRF proteins. PKIm was microinjected into cells 5 h after microinjection of a plasmid encoding HAP/SRF protein. When the localization of the expressed protein was examined 15 min later, we observed a complete loss of nuclear localization of the expressed tagged SRF protein (Fig. 3G and H). In the two double-injected cells (identified by the presence of both mouse antibodies against the HAP tag and the rabbit marker antibodies injected with PKIm, marked by arrows in Fig. 3G and H), only a cytoplasmic distribution of the expressed HAP/SRF protein is detected after 15 min. In contrast, the two adjacent cells, which have been injected only with the HAP/SRF wild-type plasmid, show a strong nuclear staining resulting from the nuclear accumulation of the expressed tagged SRF. As a control, cells injected with only rabbit marker antibody show no staining, effectively...
FIG. 3. SRF distribution after modulation of A-kinase activity. In order to examine SRF distribution after A-kinase activation, quiescent Hs-68 fibroblasts plated on glass coverslips were treated with forskolin (30 μM) and MIX (0.5 mM). After a 20-min exposure to the drugs, cells were fixed with formalin and analyzed for the distribution of SRF and actin as described in Materials and Methods. Shown are immunofluorescence micrographs of quiescent untreated cells (A and B) or quiescent cells treated with drugs for 20 min (C and D) showing actin (A and C) or SRF (B and D) distribution. An area of growing cells containing a prophase cell (marked by an arrow) is shown (E and F); panel E shows DNA staining with Hoechst, and panel F shows endogenous SRF distribution. Growing Hs-68 cells were injected with wild-type (wt) HAP/SRF plasmid. Five hours later, cells were re injected with a solution of PKIim containing rabbit marker antibody and fixed after 1 h, after which they were stained for rabbit marker antibody (G) and HAP/SRF localization (H). Arrows represent the double-injected cells. Bars = 10 μM.
excluding the possibility that cross-reactivity during the immuno-fluorescence staining technique could account for these observations (data not shown). These data, in addition to showing that inhibiting A-kinase prevents nuclear translocation of neosynthesized SRF, also demonstrate that inhibition of A-kinase results in the exit from the nucleus of the SRF already accumulated in the nuclear compartment during the previous 5 h of expression. Indeed, 15 min after PKIm injection, little or none of the expressed HAP/SRF proteins remained in the nucleus. Moreover, no SRF proteins are detected in the nucleus for as long as PKIm continues to be active (i.e., up to 4 to 5 h after its injection), confirming that nuclear import of neosynthesized SRF was also prevented by inhibiting A-kinase. Our observation of the effect of A-kinase inhibition on SRF nuclear retention and/or exit differs from our previous observations with MyoD protein. In the latter case, inhibition of A-kinase had no effect on the nuclear localization of the MyoD proteins already present in the nucleus.

The rate of import by SRF-NLS is dependent upon A-kinase activity. In light of these effects of A-kinase activation or inhibition on SRF distribution, we questioned whether the nuclear import of SRF-NLS-conjugated IgGs was directly modulated by A-kinase. To address this, solutions containing SRF-NLS-conjugated IgGs, inert mouse marker antibodies, and either the catalytic subunit of A-kinase (0.1 mg/ml) or the specific inhibitory peptide PKIm (5 \times 10^{-5} M) were injected into the cytoplasm of cells.

In order to have the minimum basal A-kinase activity, quiescent cells were used for injection of the catalytic subunit of A-kinase and the NLS-conjugated IgGs. As shown in Table 1, these cells have approximately two-fold less cAMP than those serum-stimulated cells. Cells were fixed after 30 min, a time when the distribution of injected peptide-IgG complexes was totally nuclear in serum-stimulated growing cells. In correlation with the observation that the intracellular levels of A-kinase are lower in quiescent cells, we observed that the kinetics of nuclear translocation were slower in quiescent than in proliferating cells. Indeed, when quiescent cells injected only with the SRF-NLS-IgG conjugate were fixed after 30 min (Fig. 4a, panel A), we observed approximately equivalent cytoplasmic and nuclear distributions of the IgG-conjugated peptide (panel B). Comparison of this distribution in quiescent cells with that observed 30 min after injection in growing cells (compare Fig. 4a, panels A and B, with Fig. 1c, panels C and D) shows that in the latter case, the nuclear accumulation of injected SRF-NLS-IgG is almost complete by 30 min. In a similar manner, when the quiescent cells were co-injected with the catalytic subunit of A-kinase and NLS-IgG conjugate (Fig. 4a, panel C), a predominant nuclear location of the IgGs was observed by 30 min (panel D). The residual cytoplasmic staining in panel D is completely lost when cells are injected with a higher concentration of A-kinase (0.5 mg/ml) (data not shown). However, as reported previously (24), high levels of A-kinase result in morphological changes in cells which may interfere with our assessment of cytoplasmic localization. A similar result was also obtained by treating the injected cells with forskolin to activate A-kinase (data not shown). This result shows that elevation of intracellular A-kinase levels significantly increases the rate of nuclear import of SRF-NLS-conjugated IgGs.

To examine the effect of inhibiting A-kinase activity on the nuclear import by SRF-NLS, growing cells were co-injected with SRF-NLS conjugate and PKIm and fixed 1 h after micro-injection, a time sufficient for the conjugated IgG to localize into the nucleus. As shown in Fig. 4b, when injected in the presence of the inhibitory peptide PKIm (panel A), the SRF-NLS-conjugated IgGs are found exclusively in the cytoplasm (panel B), showing the requirement for A-kinase activity in the nuclear transport by SRF-NLS. This effect was specific for A-kinase, since injection of a noninhibitory peptide, PKIm(15-24) (8) (Fig. 4b, panel C), had no effect on the nuclear localization of SRF-NLS (panel D). Moreover, inactivation of A-kinase through coinjection of the regulatory subunit of A-kinase inhibited nuclear transport of SRF-NLS-conjugated IgGs (Fig. 4b, panels E and F) in a similar manner. In contrast, specific inhibition of Ca^{2+}-phospholipid-dependent protein kinase through microinjection of another specific inhibitory peptide, C-PKI (19), did not result in any alteration in the nuclear import of SRF-NLS-conjugated IgGs (data not shown).

These results clearly show that A-kinase activity is required for the nuclear import of IgG conjugated to SRF-NLS and that a reduced rate of import by the SRF-NLS sequence is observed when the endogenous level of A-kinase is low, i.e., in quiescent cells.

**Mutation of the SRF-NLS A-kinase phosphorylation site does not affect its nuclear import activity.** Since A-kinase activity seems to be acutely involved in the active nuclear import of both SRF-NLS-conjugated IgGs and SRF protein, we examined the implication of direct phosphorylation of the putative A-kinase site previously identified in close proximity to the basic residues in the SRF-NLS sequence (20). To examine this process, synthetic peptides in which the two phosphate acceptor amino acids (serines 101 and 103) were substituted for either a nonphosphorylatable residue, alanine (SRF-NLSAla), or a residue mimicking a phosphorylation charge, aspartic acid (SRF-NLSAsp), were synthesized. In vitro phosphorylation assays using A-kinase confirmed that these peptides were not phosphorylatable (data not shown).

Peptides were chemically coupled to rabbit IgGs, and the efficiency of nuclear import of the conjugate was tested by microinjection. Both SRF-NLSAla and SRF-NLSAsp peptides translocate the conjugated rabbit IgGs into the nucleus as efficiently as does the wild-type SRF-NLS peptide. In addition, no significant differences in the nuclear import kinetics could be detected between these three peptides. However, coinjection of the A-kinase inhibitor peptide PKIm with any of these peptides effectively inhibited nuclear import and resulted in the cytoplasmic retention of the peptide-IgG conjugate, as observed with the wild-type SRF-NLS peptide (data not shown). To confirm these observations, we constructed plasmids coding for mutant forms of the whole SRF protein in which these key phosphoacceptor amino acids were substituted for nonphosphorylatable alanine or aspartic acid. Mutant plasmids in which the two serines 101 and 103 have been substituted with either alanine (HAP/SRF-Ala101-103) or aspartic acid (HAP/SRF-Asp101-103) were injected in the nuclei of growing cells. After 5 h, cells were fixed and stained for HAP/SRF localization by immunofluorescence. Both mutant forms of the protein localize into the nuclei, as observed with the wild-type plasmid (data not shown).

### Table 1. Concentration of intracellular cAMP in quiescent cells and after 30 min of serum stimulation or forskolin treatment

<table>
<thead>
<tr>
<th>Cell line</th>
<th>cAMP concn in quiescent cells (μM)</th>
<th>Serum-stimulated cells</th>
<th>Forskolin-treated cells</th>
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<tr>
<td>Hs-68</td>
<td>1.32</td>
<td>2.2</td>
<td>4.92</td>
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<td>REF-52</td>
<td>1</td>
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*Expressed as fold increase in cAMP levels over the levels measured in quiescent cells and averaged from three independent series of experiments conducted as described in Materials and Methods.
**Peptide (SRF–NLS)–IgG**

**a**

Injected cells & IgG localization

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<td>![Image A]</td>
<td>![Image B]</td>
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peptide (SRF–NLS)–IgG

30 min

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<td>![Image C]</td>
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peptide (SRF–NLS)–IgG

+ A–kinase

30 min

**b**

Injected cells & IgG localization

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<td>![Image E]</td>
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Peptide – IgG

+ PK11

60 min

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<td>![Image G]</td>
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Peptide – IgG

+ FK15–24

60 min

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Peptide – IgG

+ RH

45 min
Taken together, these results show that the requirement for A-kinase activity in the SRF-NLS-mediated nuclear transport of either NLS-IgG conjugate or overexpressed tagged SRF protein does not operate through the direct phosphorylation of the A-kinase sites present in the SRF-NLS sequence.

Inhibition of A-kinase activity ubiquitously affects the process of translocation. Inhibition of A-kinase seems to affect the nuclear translocation of SRF by a mechanism which does not involve direct phosphorylation of SRF itself. This observation, together with our previous finding of an indirect effect of A-kinase on the nuclear transport of MyoD (55), led us to examine if a requirement for A-kinase activity operates in a general mechanism in the nuclear import of all proteins. We therefore investigated the effect of inhibiting A-kinase activity on the import of SV40-NLS, since this NLS has served as a model sequence in the analysis of the process of nuclear import. Growing Hs-68 cells were co-injected with SV40-NLS-conjugated IgGs and either the inhibitor peptide PKIm (Fig. 5A and B), a noninhibitory peptide, PKIm(15-24) (panels C and D), or the A-kinase regulatory subunit RII (panels E and F). One hour after injection, in cells injected with either PKIm (Fig. 5A) or the RII subunit (panel E), the SV40-NLS-conjugated IgGs were exclusively localized in the cytoplasm (panels B and F), clearly showing the requirement for A-kinase activity in the nuclear transport by SV40-NLS. Controls using the inactive form of protein kinase, PKI(15-24) (Fig. 5C), showed no modification of the nuclear localization of SV40-NLS (panel D). Since SV40-NLS (PKKKRK) does not contain any plausible phosphorylation sites, these observations further illustrate that the modulation of nuclear import by A-kinase does not involve direct phosphorylation of the imported nuclear protein. As such, A-kinase activity may be required to phosphorylate other components of the nuclear import machinery.

To extend this observation, we have examined the effect of inhibiting A-kinase activity on the nuclear accumulation of cyclin A, a nuclear protein that does not contain any potential phosphorylation sites for A-kinase. Purified human cyclin A protein (17) was injected into the cytoplasm of Hs-68 cells during the G1 phase together with either inert mouse marker antibody alone, PKIm, or inactive PKIm(15-24). Cells were fixed 30 min thereafter and stained for the presence and localization of injected cyclin A and marker antibody. As described before (17), 30 min after injection, the injected cyclin A protein is found in the nuclei. In contrast, inhibition of A-kinase activity with PKIm restricted the cyclin A protein staining to the cytoplasm, showing that A-kinase activity is also required for cyclin A nuclear localization. The microinjection of PKIm(15-24), however, had no effect on the nuclear localization of cyclin A (data not shown). Taken together, these results support the requirement of A-kinase in a general mechanism of nuclear translocation control.

DISCUSSION

In this report, we show that the active nuclear import of SRF is directed by a unique NLS located within the N-terminal region of the protein, outside the DNA-binding domain. The efficiency of nuclear translocation of SRF is modulated by the level of A-kinase activity without involving direct phosphorylation of SRF by A-kinase. Rather, A-kinase forms part of a more general mechanism of nuclear translocation control, since the nuclear transport of both the SV40-NLS-conjugated IgGs (an NLS sequence without any phosphorylation sites) and cyclin A protein (which contains no potential A-kinase phosphorylation sites) is also prevented by inhibition of A-kinase. Taken together, our data show that active nuclear targeting of proteins through NLS involves a mechanism which requires the activity of A-kinase.

SRF contains an active NLS outside its DNA-binding domain. SRF is a constitutive nuclear protein throughout the cell cycle in various mammalian cell lines (13), having a location consistent with its function as a transcriptional regulator. Since its apparent molecular mass (67 kDa) is too great to permit random diffusion through the nuclear pore, we would anticipate there to be one or more sequences responsible for nuclear localization within SRF. Through microinjection, we have ascertained that only one of the four clusters of basic amino acids present along the SRF sequence acts in the nuclear localization of SRF by A-kinase. Rather, A-kinase forms part of a more general mechanism of nuclear translocation control, since the nuclear transport of both the SV40-NLS-conjugated IgGs (an NLS sequence without any phosphorylation sites) and cyclin A protein (which contains no potential A-kinase phosphorylation sites) is also prevented by inhibition of A-kinase. Taken together, our data show that active nuclear targeting of proteins through NLS involves a mechanism which requires the activity of A-kinase.

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of SRF. This NLS spans from amino acids 95 to 100 and is highly efficient in promoting nuclear transport. It functions as effectively as the SV40 large-T antigen NLS sequence with respect to the rate of nuclear import when coupled to IgG, even when as few as one or two NLS peptides are coupled to an IgG molecule. This high level of efficiency is consistent with the fact that SRF contains only one functional NLS and previous reports that there is a direct link between the number of NLS present in a given protein, their relative efficiencies, and the extent of nuclear import (44).

The nuclear localization domain of SRF we have identified (amino acids 95 to 100) is present in a portion of the protein exclusive to SRF, since it is not found in the various members of the SRF-related family (human and Xenopus MEF-2, RSRCF4, RSRCF9, and RSRCF2) (3, 40) or the yeast homologues (MCM1 and ARG80) (54). As such, the nuclear transport mechanism of SRF may be implicated in specific aspects of SRF protein function, particularly those processes in which rapid nuclear localization would be highly important. For example, SRF has been implicated in the rapid down regulation of c-fos, an event thought to involve newly synthesized SRF (36). Clearly, under such circumstances, when the rate of nuclear import is critical to the temporal regulation of cellular fos levels, the rapid nuclear targeting of SRF would be of importance. Finally, it is interesting that SRF-NLS does not match with either the single-motif consensus sequence (16) or the bipartite consensus drawn previously from the identification of other NLS (5). This raises the possibility that such a hybrid structure may represent another, third class of NLS, the efficiency and fidelity of which are similar to those of the other two classes.

The process of nuclear translocation requires A-kinase. Various reports have implied that nuclear localization may be modulated by specific variations in kinase activity, with phosphorylation sites often found adjacent to NLS and in some cases modulating their efficiency. In particular, A-kinase has been implicated in modulation of the subcellular localization for a variety of transcription factors. Indeed, a consensus site for A-kinase is found near the NLS in several members of the rel family. Mutation of this site to a nonphosphorylatable residue demonstrated that phosphorylation by A-kinase was implicit in the translocation of c-rel from the cytoplasm to the nucleus (36). The nuclear localization of at least four other proteins appears to be positively regulated by A-kinase. The transcription factor NFIL-6 binds to the SRE of the c-fos promoter in response to forskolin treatment. This inducible binding correlates with direct increases in phosphorylation and nuclear localization (33). The nuclear accumulation of the c-fos protein requires continuous stimulation by growth factors in a cAMP-dependent manner (45). In addition, the activation and nuclear translocation of the Drosophila Dorsal gene product requires phosphorylation by A-kinase (39). Recently, we have shown that the nuclear import of MyoD is positively modulated by A-kinase through a mechanism independent of MyoD phosphorylation (55). Here, we have shown that A-kinase-dependent phosphorylation is a necessary event in the process of nuclear localization of SRF. In addition, two new and important features concerning this requirement for A-kinase activity have been demonstrated in this study. Firstly, phosphorylation of SRF by A-kinase is not involved in determining the cytolocal of SRF, even though the site KRSLS present in SRF-NLS is a potential phosphorylation site for A-kinase. Secondly, the role of A-kinase in the mechanism of nuclear translocation described here is part of a more general process and not exclusive to SRF. This last point is clearly illustrated by the consequences of A-kinase inhibition on the nuclear translocation of both cyclin A and the SV40-NLS-conjugated IgGs.

That the blocking of nuclear transport is truly the consequence of A-kinase inhibition is demonstrated by the reproducibility of these results by using two different approaches. Nuclear transport was impeded not only by microinjection of the inhibitor peptide PKim, which might have interfered with some events in the nuclear transport process other than inhibition of A-kinase, but also by the specific regulatory subunit of A-kinase, RII, effectively ruling out possible extraneous effects of PKim. In addition, we have shown that nuclear translocation occurs at a lower rate under two different physiological conditions when the level of cAMP (and consequently of A-kinase activity) was reduced during the cell cycle. We have shown this level to be at its lowest in quiescent cells, being activated 1.5- to 2-fold upon serum stimulation (Table 1) and reduced again at the onset of mitotic induction (23). This latter report implied that a basal level of A-kinase is maintained throughout the cell cycle and required to drop to enable cells to transit from the interphase state to mitosis. We have shown that the rate of nuclear import of either SRF-NLS- or SV40-NLS-conjugated IgGs is notably reduced in quiescent cells (Fig. 1 and 4) and that an increase in cytoplasmic staining for endogenous SRF is clearly detectable in cells entering prophase (Fig. 3). Moreover, a partially cytoplasmic distribution for normally nuclear proteins (such as proliferating cell nuclear antigen, c-myc, and c-fos) has been previously observed in quiescent cells (57). Since it has been reported before that cAMP levels are reduced in quiescent cells (60) and we show here the same result for two different cell lines (Table 1), these data further support the link between a low level of A-kinase activity and a reduced efficiency in nuclear import.

In addition to a requirement for A-kinase activity in the active nuclear translocation of proteins, our data show that inhibition of A-kinase results in the rapid exit of SRF from the nucleus. This effect differs from what we observed previously with MyoD protein, which remained in the nucleus upon microinjection of A-kinase inhibitors (55), suggesting that the two processes of nuclear import and nuclear retention or exit may be functionally distinguished. However, a more detailed analysis of the implication of A-kinase in the mechanism of nuclear retention or exit of MyoD and SRF would be required to draw a conclusion about this process.

A-kinase-dependent process is a more general mechanism modulating nuclear import. SV40-NLS was one of the first NLS to be identified and has subsequently been used to model the process of nuclear translocation. Since SV40-NLS does not contain any phosphorylation sites (serine, threonine, or tyrosine), our observations using RII or PKim to prevent nuclear translocation driven by SV40-NLS definitely establish that A-kinase activity is required in the general mechanism which brings about active nuclear import.

The present study provides the first evidence for the general implication of a protein kinase, namely, A-kinase, in the control of cytonuclear translocation of proteins. Whether this implication of A-kinase control reflects a direct effect on the import process remains to be shown, since we cannot exclude the possibility that A-kinase may act through another kinase or phosphatase, itself directly implicated in the control of the import machinery proteins. Nuclear protein import is a multi-step process that results in transport of the protein from its cytoplasmic site of synthesis into the nucleus (48). An early step in nuclear protein import involves the specific recognition of the NLS in the protein by NBP5, followed either by the transport of this complex through nuclear pores into the nucleus in an ATP-dependent manner or by the interaction of

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this complex with the pores, with subsequent translocation of the nuclear protein into the nucleus and recycling of the cytosolic receptor. NPBs have been characterized in various organisms (26, 30, 46, 47, 49, 50, 51). At least two of them, NBP70 and Nopp140, are phosphorylated in vivo, an event required for their interaction with NLS (31, 51). In addition, by examining NBP sequences, one can easily identify several phosphorylation sites for different kinases, including A-kinase, again supporting the possibility that A-kinase-dependent phosphorylation may control their activity (31). In addition, some proteins present in the nuclear pore may also be targets for A-kinase regulation (4). Therefore, different hypotheses can be proposed to account for the effect of A-kinase on nuclear transport. A-kinase-dependent phosphorylation could conceivably modulate components of the nuclear pore complex, NPBs, and/or some regulatory kinase or phosphatase. An important question to be addressed is the determination of the potential target(s) involved in the A-kinase-dependent nuclear localization process of SRF and other actively transported nuclear factors. The identification of SRF-NLS is an important step in this direction, since the rapid nuclear translocation of this protein may be a useful model. Experiments to elucidate the nature of proteins potentially interacting with SRF-NLS and SV40-NLS are currently under way.

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REFERENCES


