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Nuclear Import of c-Jun Is Mediated by Multiple Transport Receptors*[S]

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c-Jun and c-Fos are major components of the transcriptional complex AP-1. Here, we investigate the nuclear import pathway(s) of the transcription factor c-Jun. c-Jun bound specifically to the nuclear import receptors importin β, transportin, importin 5, importin 7, importin 9, and importin 13. In digitonin-permeabilized cells, importin β, transportin, importin 7, and importin 9 promoted efficient import of c-Jun into the nucleus. Importin α, by contrast, inhibited nuclear import of c-Jun *in vitro*. A single basic region preceding the leucine zipper of c-Jun functions as a nuclear localization signal (NLS) and was required for interaction with all tested import receptors. *In vivo*, nuclear import of a c-Jun reporter protein lacking the leucine zipper strictly depended on this NLS. In a leucine zipper-dependent manner, c-Jun with mutations in its NLS was still imported into the nucleus in a complex with endogenous leucine zipper proteins or, for example, with cotransfected c-Fos. Together, these results explain the highly efficient nuclear import of the transcription factor c-Jun.

Nuclear transport of proteins is mediated by soluble receptor molecules belonging to the importin β superfamily. These proteins, collectively referred to as karyopherins or, depending on the direction of transport, importins and exportins, are characterized by tandem copies of HEAT repeats (for Huntingtin, elongation factor 3, protein phosphatase 2A, TOR1) that provide an interaction surface for many different binding partners. HEAT repeats are ~40 amino acids in length and adopt a helix-turn-helix conformation with anti-parallel helices. The 18–20 HEAT repeats result in a superhelical overall structure of the transport receptors with a high degree of flexibility, facilitating the binding to a large variety of different proteins (for review, see Refs. 1–2). Common interaction partners of all karyopherins are the small GTP-binding protein Ran as well as certain nucleoporins, components of the nuclear pore complex. In nuclear export, RanGTP is an integral component of the transport complex. In nuclear import, by contrast, RanGTP functions in the dissociation process of import complexes in the nucleus (3), as binding of cargo molecules and RanGTP to importins is considered to be mutually exclusive. A high concentration of nuclear RanGTP, as required for the formation of export complexes and dissociation of import complexes, results from the activity of the chromatin-associated guanosine nucleotide exchange factor for Ran, RCC1. In the cytoplasm, on the other hand, the GTPase-activating protein RanGAP together with the Ran-binding protein RanBP1 promote GTP hydrolysis on Ran. RanGDP is then imported into the nucleus by a dedicated transport factor, NTF2 (for review, see Refs. 4 and 5).

Importins recognize their cargo molecules via certain nuclear localization signals (NLSs).[2] The "classic" NLS is characterized by short stretches of basic amino acids, typically lysine residues, occurring either as a single or a bipartite motif. Both motifs interact with the adapter protein importin α, which in turn binds to the *bona fide* import receptor, importin β (for reviews, see Ref. 2 and 4–6). The importin α/β complex, thus, functions as an entity to import hundreds of different proteins into the nucleus. Besides importin β, ~20 members of the importin β superfamily have been identified, about 10 of which appear to function in nuclear import. The best characterized receptor besides importin β itself is transportin, which binds to an NLS of a 38-amino acid domain in the heterogeneous nuclear ribonucleoprotein A1 protein, the M9-sequence (7). Transportin is also involved in nuclear import of ribosomal proteins (8), the nucleoporin Nup153 (9), and the Rev protein of the human immunodeficiency virus (10). Very recently we described transportin as a major importin for the transcription factor c-Fos (11). Furthermore, the superfamily includes importin 5, importin 7, and importin 9, which transport ribosomal proteins and core histones into the nucleus (8), importin 8, which mediates nuclear import of a protein of the signal recognition particle (12), importin 11, importing the ubiquitin conjugating enzyme UbcM2 (13), and importin 13, which functions as an import receptor for the small ubiquitin-like modifier-conjugating enzyme Ubc9 (14). These importins do not use adapter proteins for nuclear import but bind directly to their cargo molecules. Curiously, importin β can function in both modes either using the adapter protein importin α or not. Examples for proteins that directly interact with importin β are the human immunodeficiency virus-Rev protein, the parathyroid hormone-related protein (PTHrP) (15), cyclin B1 (16), the sterol regulatory element-binding protein 2 (SREBP-2) (17), and the transcription factor c-Fos (11).

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

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[2] The abbreviations used are: NLS, nuclear localization signal; AP-1, activator protein-1; BSA, bovine serum albumin; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HA, hemagglutinin; HEAT, huntingtin, elongation factor 3, protein phosphatase 2A, TOR1; LZ, leucine zipper; MBP, maltose-binding protein; WGA, wheat germ agglutinin; IBB, importin β binding.

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c-Fos and c-Jun are components of activator protein-1 (AP-1), one of the best characterized mammalian transcription factors (for reviews, see Refs. 18 and 19). AP-1 functions as a dimer of basic leucine zipper (LZ) proteins, where α-helices of the individual proteins interact mainly via leucine residues at every seventh position of the leucine zipper (20). A structural analysis of a heterodimer of the basic LZ domains of c-Jun and c-Fos bound to DNA revealed a pair of extended α-helices interacting at one end with each other via the leucine zipper and at the opposing basic end with the target DNA (21, 22). Upon DNA binding, AP-1 is known to induce transcription of a large variety of genes (for review, see Ref. 23).

Compared with c-Fos, rather little is known about the nuclear import characteristics of c-Jun. The basic stretch preceding the leucine zipper of c-Jun, which is involved in DNA binding, has also been shown to function as an NLS (24, 25). In transfection experiments, c-Jun promoted nuclear import of c-Fos in dependence on their leucine zippers (26), suggesting that the AP-1 components can be transported into the nucleus as heterodimers. Using purified components, c-Jun has been shown to interact with intermediate affinities with importin α and importin β (27). Nuclear import of c-Jun in permeabilized cells with importin β or alternative transport receptors has, however, not been demonstrated. We now use digitonin-permeabilized cells to show that not only importin β, but also transportin, importin 7, and importin 9 can function as efficient import receptors for c-Jun. In binding studies these proteins as well as importin 5 and importin 13 specifically interacted with c-Jun. Binding to all tested importins as well as nuclear import in living cells was abolished when the basic NLS of c-Jun was mutated, suggesting that a single stretch of basic amino acids provides the binding surface for multiple transport receptors. Together, these results demonstrate the high level of promiscuity of c-Jun with respect to nuclear import receptors.

EXPERIMENTAL PROCEDURES

Cell Culture—Adherent HeLa P4 cells (28) were grown in Dulbecco's modified Eagle's medium (Invitrogen) containing 4.5 g/liter glucose, 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Molecular Clones—The plasmid coding for His-tagged c-Jun from rat was described previously (29). For expression of His-maltose-binding protein (MBP)-tagged proteins, the full-length c-Jun sequence or fragments thereof were amplified by PCR using appropriate primers and cloned into the NcoI/KpnI sites of pETM41a (obtained from Ario de Marco, EMBL, Heidelberg, Germany).

Point mutations were introduced by site-directed mutagenesis using the oligonucleotide pairs 5′-GCCTCCAAGTGCGCGAAAGCGAAGCTGGAGCGG-3′/5′-CCGCTCCAGCTTCGCTTTCGCGCACTTGGAGGC-3′ (introducing the R273A/R275A mutation into the c-Jun wild-type sequence) and 5′-GCCTCCAAGTGCGCGGCGGCGGCGCTGGAGCGGATC-3′/5′-GATCCGCTCCAGCGCCGCCGCCGCGCACTTGGAGGC-3′ (introducing the K274A/K276A mutation into the R273A/R275A mutation).

For cloning into the reporter construct pEGFP-glutathione *S*-transferase (pEGFP-GST; kindly provided by Sonja Neimanis, Zentrum für Biochemie und Molekulare Zellbiologie, Göttingen), c-Jun fragments coding for amino acids 1–279 (ΔLZ) or 1–334 (full-length) were PCR-amplified from pETM41a vectors (wild type or mutated) using appropriate oligonucleotides containing EcoRI and Acc65I restriction sites. The coding sequence for rat c-Fos was introduced into the mammalian expression vector pEF-HA containing an N-terminal hemagglutinin tag (30) (kindly provided by Saskia Hutten, Zentrum für Biochemie und Molekulare Zellbiologie, Göttingen, Germany).

Protein Purification—The purification of His-c-Jun was largely performed as described (29). Briefly, BL21-DE3 cells transformed with the plasmid coding for His-tagged c-Jun from rat (accession number NM_021835) were induced to express the protein with 0.5 mM isopropylthio-β-D-galactoside at 25 °C for 3 h. Bacteria were lysed in buffer B (50 mM Tris, pH 7.4, 150 mM NaCl, 6 M guanidinium hydrochloride), and insoluble components were pelleted by centrifugation at 100,000 × *g* for 45 min. The supernatant was incubated with nickel-nitrilotriacetic acid-agarose (Qiagen) for 1.5 h at 20 °C. Beads were washed with buffer B, and bound proteins were eluted with 300 mM imidazole in buffer B and dialyzed extensively against phosphate-buffered saline containing 5% glycerol, 1 mM dithiothreitol, and 1 μg/ml each of aprotinin, leupeptin. and pepstatin.

All His-MBP-tagged proteins were expressed in BL21-DE3 upon induction with 0.5 mM isopropylthio-β-D-galactoside at 20 °C for 3 h. Bacteria were lysed in buffer A (50 mM Tris, pH 8, 250 mM NaCl, 2 mM $MgCl_2$, 10% glycerol) containing 1% Triton X-100, 4 mM β-mercaptoethanol, 0.4 μg/ml lysozyme, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin. Insoluble components were pelleted by centrifugation at 100,000 × *g* for 45 min, and the supernatant was incubated with nickel-nitrilotriacetic acid-agarose (Qiagen) for 1.5 h at 4 °C. Beads were washed with buffer A containing 0.1% Triton X-100 and β-mercaptoethanol and protease inhibitors as above. Bound proteins were eluted with 300 mM imidazole in buffer A and dialyzed against phosphate-buffered saline containing 5% glycerol, 1 mM dithiothreitol, and protease inhibitors.

GST-IBB (11), His-importin-α (31), His-S-importin β (32), His-transportin (33), His-importin 5 (8), His-importin 7 (34), His-importin 9 (35), His-importin 13 (14), and RanQ69L (36) were purified as described. All nuclear transport factors were dialyzed against transport buffer (20 mM Hepes, pH 7.3, 110 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM EGTA).

For binding studies RanQ69L was loaded with GTP, as described (37). Preparation of Cy3-BSA-NLS was essentially as described (38). All proteins were frozen in liquid nitrogen and stored at −80 °C.

Binding Studies—5 μg of His-MBP fusion proteins were immobilized on 20 μl of amylose beads (New England Biolabs) that had been preincubated with 10 mg/ml BSA in buffer C (50 mM Tris pH 7.4, 200 mM NaCl, 1 mM $MgCl_2$, 5% glycerol). The beads were washed and incubated with 5 μg of import receptors in 300 μl of buffer C containing 2 mg/ml BSA. Where indicated, reactions included 1.3 μM RanQ69L-GTP. After 1.5 h at 4 °C, beads were washed 3 times with buffer B. Bound proteins were

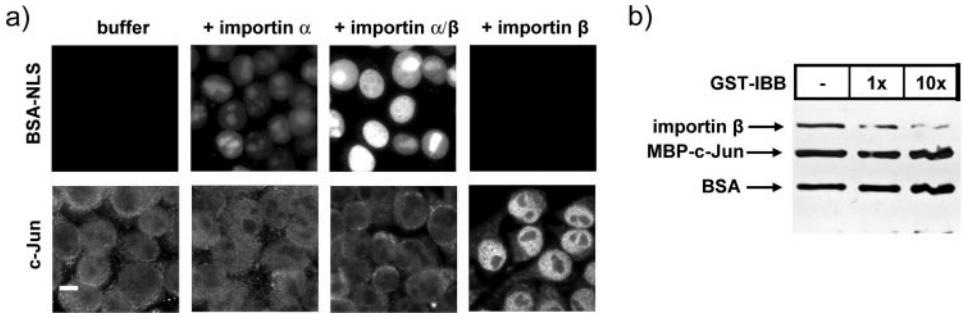

FIGURE 1. **Nuclear import of c-Jun does not depend on importin α.** *a*, permeabilized cells were incubated with the import substrates BSA-NLS or c-Jun in the presence of Ran and buffer, importin α, and importin β, as indicated. Cells were either analyzed directly (BSA-NLS) or after indirect immunofluorescence (c-Jun). The *bar* corresponds to 10 μm. *b*, MBP-c-Jun was immobilized on amylose beads and incubated with importin β alone or with an equal concentration (1×) or a 10-fold excess (10×) of GST-IBB. Interacting proteins were analyzed by SDS-PAGE followed by silver staining. BSA was used as a blocking reagent.

eluted with SDS-sample buffer and subjected to SDS-PAGE followed by silver staining.

SDS-PAGE and Western Blotting—Proteins were analyzed by SDS-PAGE and Western blotting using standard methods. Anti-penta-His (Qiagen) was used as primary antibody to detect His-tagged importins as well as His-MBP-c-Jun. Horseradish peroxidase-coupled goat anti-mouse IgG (Dianova) was used as secondary antibody. The ECL system (Pierce) was used for visualization of proteins.

In Vitro Import Assays—For nuclear import assays (39), HeLa cells were grown on 10-well slides (Erie Scientific Co.), permeabilized with 0.015% digitonin (Calbiochem) in transport buffer on ice, and washed. Where indicated, permeabilized cells were preincubated with 200 μg/ml wheat germ agglutinin (WGA; Sigma) in transport buffer for 12 min on ice. Import mixtures contained 200 nM Cy3-BSA-NLS or 500 nM of His-c-Jun, 4 μM Ran, 2 mg/ml BSA, an ATP regenerating system (40), and 500 nM His-tagged importins. After washing with transport buffer, cells incubated with Cy3-BSA-NLS were fixed with 3.7% formaldehyde and analyzed directly by fluorescence microscopy. Cells incubated with His-c-Jun were first subjected to indirect immunostaining using a rabbit anti-c-Jun antibody (Santa Cruz) as primary and donkey-anti-rabbit Alexa 488 (Molecular Probes) as secondary antibody. Cells were analyzed by fluorescence microscopy using a Zeiss Axioskop2 microscope. Pictures were processed using Adobe Photoshop.

Transfection Experiments—HeLa cells were transfected with plasmids coding for green fluorescent protein (GFP)-GST reporter proteins and anti-hemagglutinin (HA)-c-Fos using the calcium phosphate method (41). Cells were fixed with 3.7% formaldehyde and either analyzed directly by fluorescence microscopy or first subjected to indirect immunostaining using a monoclonal HA antibody (12CA5) and donkey-anti mouse Alexa 594 (Molecular Probes) to detect HA-c-Fos. For a quantitative analysis, cells were scored into the following categories: N > C (more reporter protein in the nucleus), N < C (more reporter protein in the cytoplasm), and N = C (equal distribution of the reporter protein between nucleus and cytoplasm).

RESULTS

Multiple Importins Function as Import Receptors for c-Jun—The nuclear transport factors importin α and importin β have been implicated in nuclear import of the transcription factor c-Jun. Using an enzyme-linked immunosorbent assay-based binding assay, importin α ($K_d \sim 160$ nM) and importin β ($K_d \sim 70$ nM) have been shown to interact with similar affinities with c-Jun (27). To investigate the contribution of these transport factors to nuclear import, we established an *in vitro* system using digitonin-permeabilized HeLa cells (39) for the analysis of import of c-Jun. As a control, we used the established import substrate BSA-classic NLS (the SV 40 large T antigen NLS, coupled to BSA), which uses the importin α/β heterodimer for nuclear import. For BSA-NLS, importin β alone was not sufficient for efficient nuclear import, and importin α was required in addition (Fig. 1*a*; a quantification of import is shown in supplemental Fig. S1). For c-Jun, by contrast, importin β alone was sufficient for nuclear accumulation (for controls, compare Fig. 3). Upon the addition of importin α, transport of c-Jun into the nucleus was strongly reduced (Fig. 1*a*), suggesting that importin α and c-Jun compete for binding to importin β. Indeed, importin β interacted with an immobilized fusion protein of c-Jun and the MBP (Fig. 1*b*). Binding was strongly reduced when an excess of GST-IBB containing the importin β binding domain of importin α was included in the reaction. These results clearly demonstrate a fundamental difference between the nuclear import pathways of BSA-NLS and c-Jun, as the adapter protein importin α is not required for nuclear accumulation of the transcription factor but is rather inhibitory for the reaction.

Is importin β the only importin that is able to translocate c-Jun into the nucleus, or do other members of the importin β-superfamily function as import receptors as well? To answer these questions, different importins were analyzed for binding to MBP-c-Jun. To test for specificity of the interaction, the reactions were performed in the absence or presence of RanQ69L, loaded with GTP. This Ran mutant does not hydrolyze its bound nucleotide (42) and dissociates functional importin-cargo complexes. As shown in Fig. 2, all tested importins (importin 5, importin 7, importin 9, importin 13, importin β, and transportin) interacted with immobilized c-Jun. Binding of importin 5, importin 9, importin 13, importin β, and transportin to MBP-c-Jun was strongly reduced in the presence of RanQ69L. For importin 7, only a small reduction in binding was observed when RanQ69L was included in the reaction. This may result from an intrinsically low affinity of RanGTP for His-tagged importin 7, as it was used in these experiments (34). Because importin 7 also promoted nuclear import of c-Jun (see below), we consider it unlikely that its observed binding to the transcription factor resulted from nonspecific interactions. Hence, for the tested importins, our results show the formation of specific c-Jun containing transport complexes that can (in most cases) be dissociated by RanGTP.

We next analyzed whether the individual importins are equally able to promote import of c-Jun into the nucleus of digitonin-permeabilized cells. As shown in Fig. 3, not only importin β (compare Fig. 1) but also importin 7, importin 9, and transportin allowed efficient nuclear accumulation of c-Jun at 30 °C but not at 4 °C. No or very little nuclear import of c-Jun was observed when transport receptors were omitted (Fig. 3*s*) or when importin 5 (Fig. 3*a*) or importin 13 (Fig. 3*d*) were added to the reaction. As a control for the integrity of the nuclear envelope under our experimental conditions, we included WGA in the reactions (Fig. 3, *m–r*). WGA is a lectin that binds to *O*-glycosylated nucleoporins (43) and inhibits various nucleocytoplasmic transport pathways (44, 45). As expected, WGA strongly inhibited nuclear import of c-Jun in the presence of importin 7, importin 9, importin β, and transportin. Taken together, our results demonstrate that the transcription factor c-Jun uses multiple transport receptors for nuclear import *in vitro*, including importin 7, importin 9, importin β, and transportin. These importins may, therefore, also function as physiological import receptors in living cells.

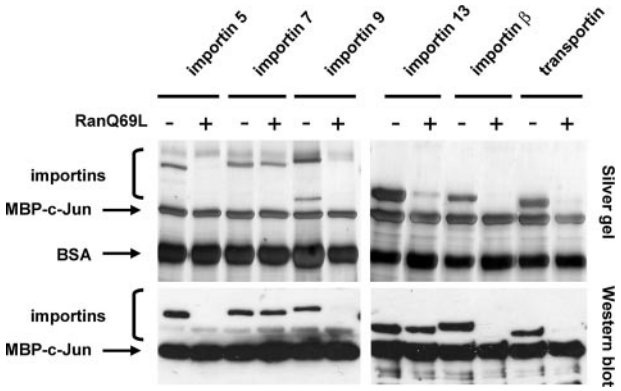

FIGURE 2. **c-Jun interacts specifically with various importins.** MBP-c-Jun was immobilized on amylose beads and incubated with importins in the absence or presence of RanQ69L, which had been loaded with GTP, as indicated. BSA was used as a blocking reagent. Interacting proteins were analyzed by SDS-PAGE followed by silver staining (*top*) or Western blotting (*bottom*). For an estimate of binding efficiencies compared with protein input, see supplemental Fig. S3.

A Single NLS Mediates Binding of c-Jun to Importins and Nuclear Import of the Transcription Factor—The basic region of c-Jun preceding the leucine zipper has been described as a major NLS of the transcription factor (24, 25). To characterize the region of c-Jun that is required for binding to the individual import receptors as identified above, we first expressed N-terminal deletion mutants of c-Jun fused to MBP. The constructs and the expressed proteins are shown in Fig. 4, *a* and *b*. The deletion mutants were immobilized on amylose beads and incubated with the importins in the absence or presence of RanQ69L that had been loaded with GTP. Deletion of 124 amino acids from the N terminus of c-Jun did not significantly change its binding to importins compared with the wild-type protein (data not shown). Similarly, deletion of 222 (Fig. 5*a*) or 249 (Fig. 5*b*) amino acids from the N terminus did not result in a significant loss of binding of any of the importins to the transcription factor. As before (compare Fig. 2), binding of importin 7 to the various c-Jun proteins was not efficiently blocked by RanQ69L, in contrast to that of the other importins. These results suggest that the first 249 amino acids of c-Jun are not required for binding of the protein to its various import receptors. In our shortest c-Jun mutant, MBP-c- Jun-(278–334), the deletion also encompasses the basic region in front of the leucine zipper of the transcription factor. None of the tested importins was able to interact with this truncated form of c-Jun (Fig. 5*c*), suggesting that the basic region is required for efficient binding.

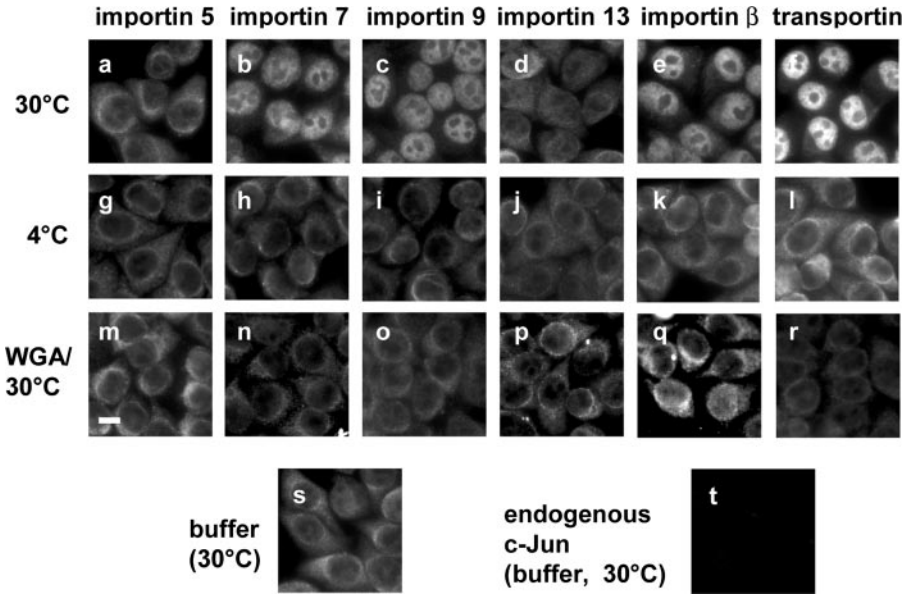

FIGURE 3. **Multiple importins promote nuclear import of c-Jun.** Permeabilized cells were incubated with recombinant c-Jun and buffer (*s*) or different importins at 30 °C (*a–f*, *m–r*) or 4 °C (*g–l*). For reactions *m–r*, cells were preincubated with WGA to block active nucleocytoplasmic transport. All reactions contained the nuclear import factor Ran. Cells were subjected to indirect immunofluorescence, detecting recombinant c-Jun. Endogenous c-Jun is detected in *t*, where the import substrate and the importins were omitted from the reaction. The *bar* in *m* corresponds to 10 μm. A quantitative analysis of this experiment is presented in supplemental Fig. S2.

To directly test the role of the basic amino acids in the region preceding the leucine zipper, we performed site-directed mutagenesis in the context of the full-length protein and expressed the double mutant MBP-c-Jun R273A/R275A and the quadruple mutant MBP-c-Jun R273A/K274A/R275A/K276A. Wild-type protein as well as the mutants were immobilized on amylose beads and incubated with importin 5, importin 7, importin 9, importin 13, importin β, or transportin. Compared with the wild-type protein, interaction of the double mutant MBP-c-Jun R273A/R275A with importin 5, importin 9, importin 13, and importin β was clearly reduced (Fig. 5*d*). Only a small or no reduction was observed for the binding of importin 7 and transportin to the double

mutant. The quadruple mutant MBP-c-Jun R273A/K274A/R275A/K276A showed almost no binding to the import receptors under our experimental conditions. These results suggest that a single NLS in c-Jun, a basic region including amino acids 273–276, is responsible for interaction with multiple importins.

We next analyzed nuclear import of c-Jun in living cells. To facilitate the analysis, the coding sequence of c-Jun was fused to a construct that expresses a fusion protein of GFP and GST. GFP-GST alone resides predominantly in the cytoplasm when expressed in mammalian cells because it is too large to enter the nucleus by passive diffusion and does not contain an active NLS (Fig. 6*a*, *lower panel*). Fusion of wild-type c-Jun to GFP-GST lead to efficient nuclear import of the resulting protein, showing that the NLS of the transcription factor is active under our experimental conditions (Fig. 6*a*, *upper panel*). Efficient nuclear import could also be observed when the fusion protein lacked the leucine zipper (ΔLZ; Fig. 6*a*, *middle panel*), suggesting that the formation of homodimers or heterodimers with endogenous proteins is not required for transport. To investigate the role of the basic NLS, we now intro-

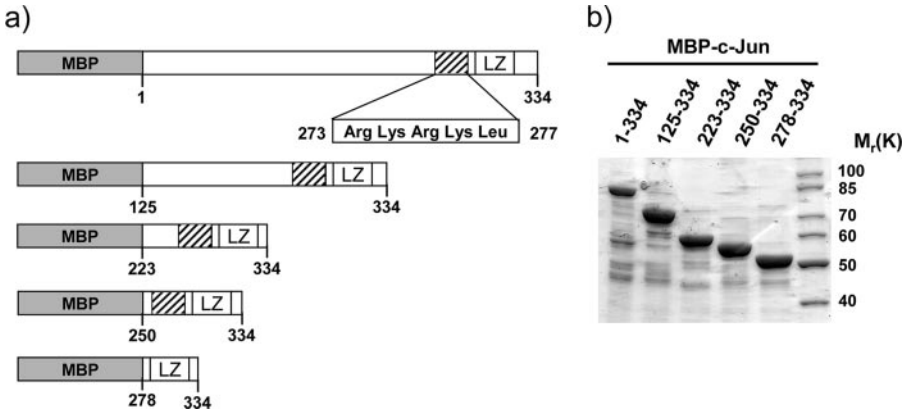

FIGURE 4. **N-terminal deletion mutants of c-Jun, fused to MBP.** *a*, schematic representation of the fusion proteins. The leucine zipper (LZ) and the basic region (*hatched area*) are not drawn to scale. *b*, proteins were expressed in bacteria, purified, and subjected to SDS-PAGE followed by Coomassie staining.

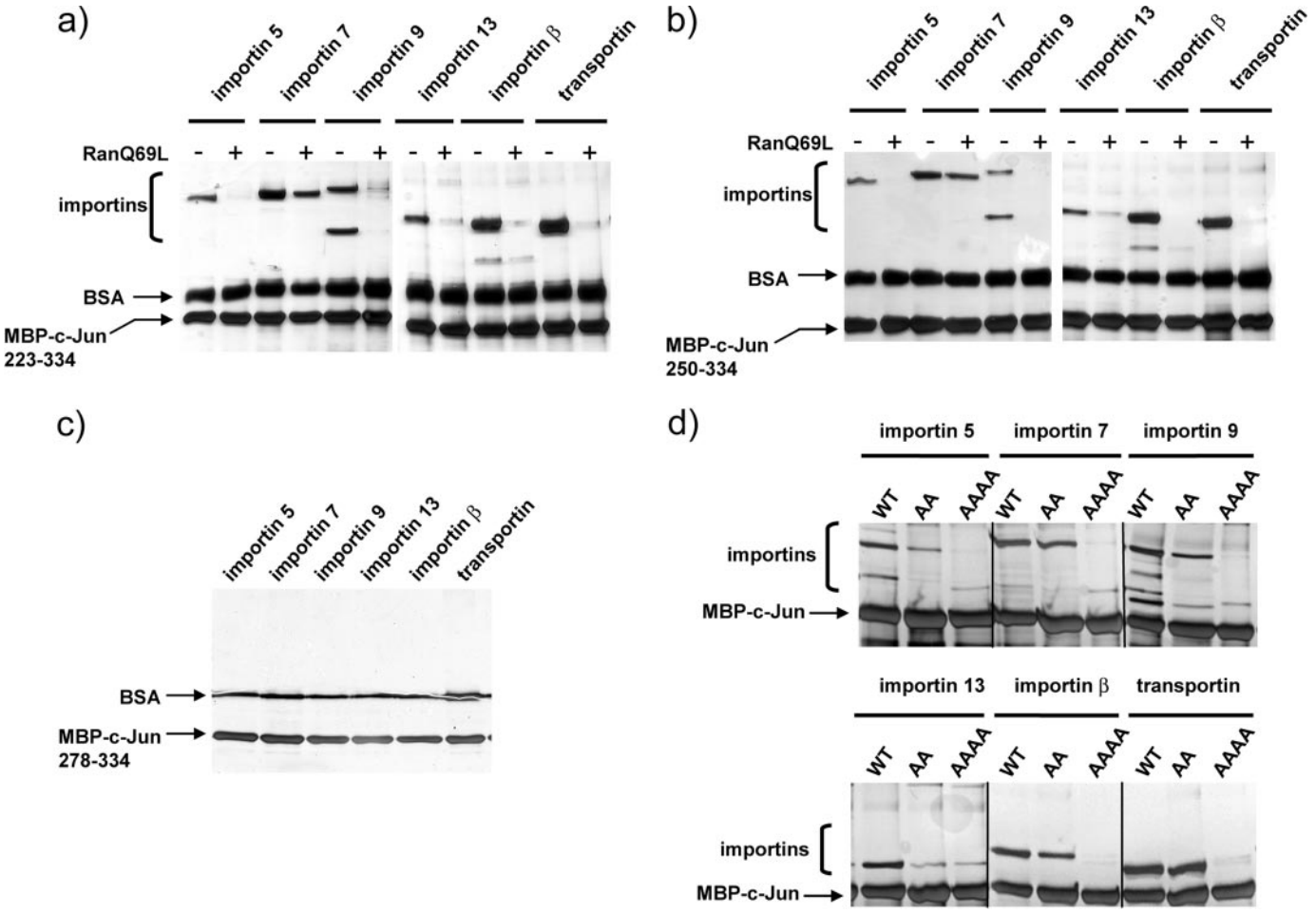

FIGURE 5. **The basic region of c-Jun preceding the leucine zipper is required for binding to various importins.** *a–c*, MBP-c-Jun fusion proteins were immobilized on amylose beads and incubated with importins in the absence or presence of RanQ69L, which had been loaded with GTP, as indicated. Specific binding of importins was observed for MBP-c-Jun-223–334 (*a*) and MBP-c-Jun-(250–334) (*b*) but not for MBP-c-Jun-(278–334) (*c*). *d*, wild-type MBP-c-Jun (*WT*), the double mutant R273A/R275A (*AA*), and the quadruple mutant MBP-c-Jun R273A/K274A/R275A/K276A (*AAAA*) were immobilized on amylose beads and incubated with import receptors, as indicated. A larger area of the gels and an input control is presented in supplemental Fig. S3. *a–d*, BSA was used as a blocking reagent. Interacting proteins were analyzed by SDS-PAGE followed by silver staining.

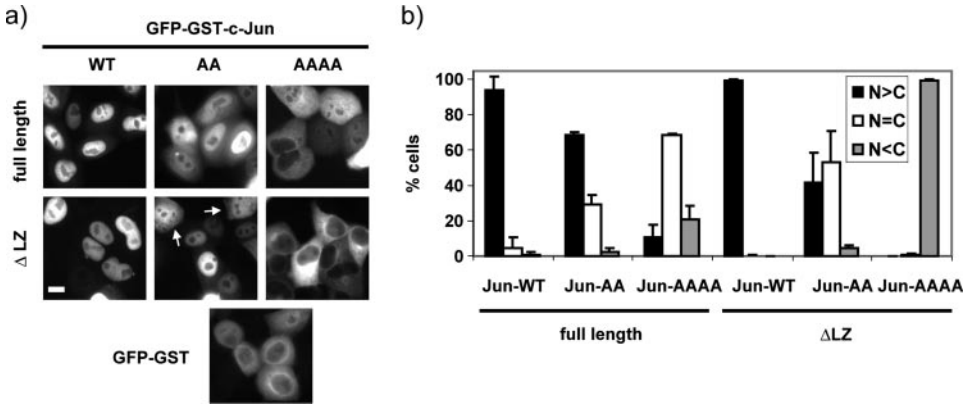

FIGURE 6. **The basic region of c-Jun is required for nuclear import *in vivo*.** *a*, HeLa cells were transfected with plasmids coding for full-length c-Jun or a fragment lacking the leucine zipper (Δ*LZ*) fused to GFP-GST. c-Jun was either wild-type (*WT*), R273A/R275A (*AA*), or R273A/K274A/R275A/K276A (*AAAA*) as indicated. Cells were analyzed by fluorescence microscopy. *Arrows* point to cells with a clear cytoplasmic signal. The *bar* corresponds to 10 μm. *b*, quantification of two independent experiments. At least 100 cells were analyzed per condition. *Bars* indicate the variation from the mean.

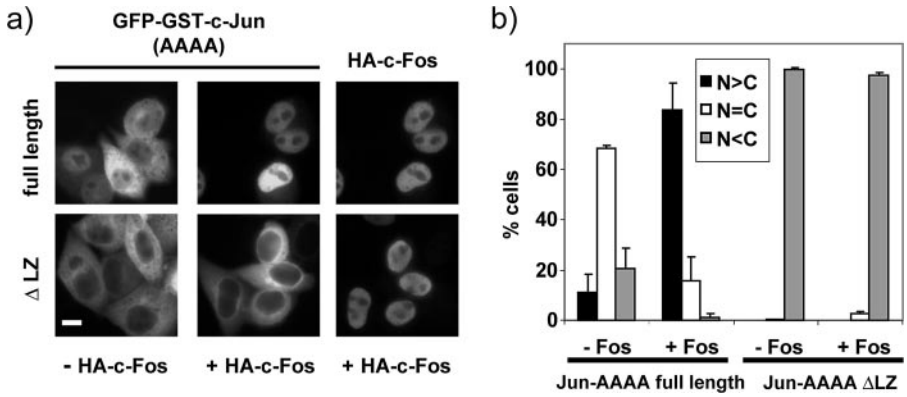

FIGURE 7. **c-Fos promotes nuclear import of c-Jun.** *a*, the quadruple mutants GFP-GST-c-Jun R273A/K274A/R275A/K276A (*AAAA*) in the context of the full-length protein or of the ΔLZ-deletion mutant were expressed in HeLa cells either with or without HA-c-Fos, as indicated. For detection of c-Fos, cells were subjected to indirect immunofluorescence using the anti-HA antibody. The bar corresponds to 10 μm. *b*, quantification of two independent experiments. At least 100 cells were analyzed per condition. *Bars* indicate the variation from the mean.

duced mutations in the basic region of c-Jun into the GFP-GST reporter proteins in the context of both the full-length protein and the ΔLZ deletion mutant. The full-length double mutant GFP-GST-c-Jun R273A/R275A was still imported into the nucleus, although to a lower extent compared with the wild type, as some fluorescence was also detected in the cytoplasm (Fig. 6*a*, *upper panel*). Cells transfected with the plasmid coding for the quadruple mutant GFP-GST-c-Jun R273A/K274A/R275A/K276A showed either no nuclear import or an equal distribution of the reporter protein between the nucleus and the cytoplasm. These results show that the basic region of c-Jun strongly affects nuclear import of the transcription factor. The quadruple mutant, which hardly binds to any of the tested importins (compare Fig. 5), could be imported into the nucleus upon heterodimer formation with endogenous basic leucine zipper containing proteins. To rule out this possibility, we also tested the double and the quadruple mutant in the context of the GFP-GST-ΔLZ-c-Jun construct, which lacks the leucine zipper. Indeed, GFP-GST-ΔLZ-c-Jun R273A/K274A/R275A/K276A (AAAA) was completely excluded from the nuclear volume (Fig. 6*a*, *lower panel*). The double mutant GFP-GST-ΔLZ-c-Jun R273A/R275A (AA) showed an intermediate phenotype, suggesting that productive interaction with some cellular importins can still occur. Fig. 6*b* shows a quantitative analysis of the data in Fig. 6*a*. These results demonstrate the importance of the basic NLS for nuclear import of c-Jun.

Finally, we directly tested the hypothesis that c-Jun can be imported into the nucleus upon heterodimerization with other LZ-containing proteins. The quadruple mutants GFP-GST-c-Jun R273A/K274A/R275A/K276A with or without the leucine zipper were transfected into cells with or without a potential binding partner, the c-Fos protein fused to a hemagglutinin tag (HA-c-Fos). As shown in Fig. 7, *a* and *b*, cotransfection of c-Fos rescued nuclear import of the c-Jun mutant, which only inefficiently entered the nucleus when expressed on its own. This rescue depended on the presence of the leucine zipper, as the c-Jun mutant lacking this dimerization domain was not imported into the nucleus either in the absence or in the presence of cotransfected c-Fos. In summary, our results point to the various pathways the transcription factor c-Jun can take to efficiently enter the nucleus.

DISCUSSION

In this study we have analyzed the nuclear import pathways of the transcription factor c-Jun in detail. Our *in vitro* assay allowed us to compare the activities of various nuclear transport factors toward import of c-Jun in permeabilized cells. The classic import receptor, the importin α/β dimer, had previously been implicated as a binding partner for c-Jun (27). The authors of this study, however, suggested that importin β alone serves as an import receptor for c-Jun. Our results now clearly show that importin β indeed binds specifically to c-Jun and is also able to import the transcription factor into the nucleus. The adapter protein importin α, however, is not required for efficient transport. The fact that importin α actually inhibited nuclear import of c-Jun and the observation that GST-IBB reduced the binding of importin β to the transcription factor suggest that importin α competes with c-Jun for binding sites on importin β. Alternatively, binding of importin α to importin β could result in structural changes in importin β, preventing c-Jun from binding to another site. It will be interesting to analyze the region of

importin β that is required for binding to c-Jun. For c-Fos, another component of the AP-1 complex, we could recently show that its binding site on importin β can be separated from that for its classic import cargo, importin α (11). Together, these results point to the high flexibility of importin β to accommodate a large variety of different import cargos.

Besides importin β, a number of other, less well characterized importins function as efficient transport receptors for c-Jun, at least *in vitro*. These include importin 7, importin 9, and transportin. The last has been shown to be the major importin for c-Fos (11). In the M9-sequence of heterogeneous nuclear ribonucleoprotein A1 and other proteins, Lee *et al.* (46) have recently identified a consensus site for binding of cargo molecules to transportin containing a characteristic proline-tyrosine (PY) motif. Although such a site is also present in c-Fos, binding of this transcription factor to transportin clearly differs from that of the standard binding partner, the M9 sequence (11). In c-Jun we did not find a PY -motif that could function in transportin binding. Therefore, we consider it likely that distinct mechanisms are used for binding of c-Jun to transportin, similar to c-Fos.

An NLS has previously been identified in the basic region preceding the leucine zipper of c-Jun (24, 25). We now expand on these findings and show that this sequence is required for interaction with all importins tested in this study. This is reminiscent of the situation in the Rev protein of the human immunodeficiency virus, where a single basic NLS mediates interaction with importin β, importin 5, importin 7, and transportin (10). In c-Fos, by contrast, the NLSs that are recognized by importin β and transportin could be separated (11). Of note, binding of importin β importin 5, importin 9, and importin 13 to c-Jun was affected by a mutation of only two basic residues in the NLS of the transcription factor. For importin 7 and transportin, by contrast, four basic residues had to be changed to alanines to observe a significant reduction in binding to c-Jun. A detailed analysis of association and dissociation constants will be required to see if this behavior actually reflects differences in binding affinities.

All active importins that were identified in this study also function in nuclear import of ribosomal proteins and histones. These very abundant cargos that require an efficient nuclear import pathway are characterized by their prominent positive charges, a common feature of many nuclear proteins. Apparently, c-Jun exposes its basic NLS in a way that allows recognition by different import receptors, possibly similar to other basic proteins that use multiple importins for nuclear import. Interestingly, viral Jun (v-Jun), but not c-Jun, contains a serine residue just in front of the basic NLS (24). This residue is subject to phosphorylation, leading to inhibition of nuclear import of a reporter protein containing the v-Jun NLS. Thus, cell cycle-dependent regulation of nuclear import of v-Jun has been attributed to the introduction of a negative charge close to the basic NLS (47).

Two importins, importin 5 and importin 13, were shown to interact with c-Jun *in vitro* but did not support efficient import of the transcription factor in permeabilized cells. We cannot rule out the possibility, however, that *in vivo* these transport receptors may also be involved in nuclear import of c-Jun under certain conditions.

In our transfection experiments, a c-Jun fragment lacking the leucine zipper promoted nuclear import of a GST-GFP reporter protein, demonstrating that dimerization via the leucine zipper is not required for efficient transport. Mutation of two basic residues in the NLS lead to a small reduction of nuclear import. In this case transport might have occurred via transportin or importin 7, which both interact with this mutant in a similar way as with the wild-type protein. Nuclear import was strongly inhibited when four of the basic residues in the NLS were mutated to alanines. Constructs containing the leucine zipper, however, could be imported into the nucleus at least to some extent, suggesting that endogenous proteins can bind to the c-Jun mutant and take it into the nucleus by means of a "piggyback" mechanism. The observation that cotransfected c-Fos, an established dimerization partner for c-Jun, promotes nuclear uptake of c-Jun with mutations in its NLS further supports this observation. Taken together, our results show that c-Jun can use different pathways to travel into the nuclear compartment; first, it can interact via its own NLS with many different nuclear transport receptors, just as very abundant nuclear proteins like histones or ribosomal proteins. Hence, import of c-Jun is independent of the availability of individual importins, assuring efficient transport under all conditions. Second, c-Jun may form heterodimers with other cellular proteins containing a basic leucine zipper, *e.g.* c-Fos, and travel into the nucleus using the NLS and the interacting importins of those proteins. Formation of heterodimers of c-Jun and c-Fos has previously been demonstrated to occur in the cytoplasm (48). Import complexes of these heterodimers could actually contain more than one importin during their passage through the nuclear pore complex. It will be interesting to investigate the composition of these transport complexes in the future.

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