Nuclear Import of Creb and AP-1 Transcription Factors Requires Importin-β1 and Ran but Is Independent of Importin-α

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ABSTRACT: Although the specific role of transcription factors (TFs) is nuclear, surprisingly little is known in quantitative terms regarding the pathways by which TFs localize in the nucleus. In this study, we use direct binding assays, native gel electrophoresis, and fluorescence polarization measurements to show for the first time that the cAMP-response element binding protein (CREB) and related AP-1 and jun and fos constituents are recognized by importin β1 (Impβ) with nanomolar affinity. We reconstitute the nuclear import of these TFs in vitro, demonstrating dependence on cytosolic factors, and show that this is due to the requirement for Impβ, since antibodies to Impβ, but not to importin α (Impα), inhibit nuclear accumulation significantly. We show that Impβ is necessary and sufficient for docking of CREB at the nuclear envelope; that Ran is essential for CREB nuclear import is demonstrated by the reduction of nuclear accumulation effected by RanGTPγS but not RanGDP, and by dissociation of the Impβ−CREB-GFP complex by RanGTPγS but not RanGDP as demonstrated using fluorescence polarization assays. The results support the existence of an Impβ1- and Ran-mediated nuclear import pathway for CREB and related constitutively nuclear TFs, which is Impα-independent and thus distinct from import pathways utilized by inducible TFs.

Transcription factors (TFs),[1] many of which are expressed in a cell-specific or developmentally regulated manner, activate transcription either constitutively or in a regulated fashion. TFs of the basic leucine zipper (bZIP) family bind specific DNA sequences as homo- or heterodimers, interacting with DNA through a surface provided by a region of basic amino acids immediately preceding the leucine zipper domain (*1*). The bZIP TF CREB (cAMP-response element−CRE−binding protein) is responsible for activating CRE-driven transcription (*2*), regulation of its activity occurring through phosphorylation at Ser^{133} by cAMP-dependent protein kinase or Ca^{2+}/calmodulin dependent protein (*3*, *4*). CREB's biological role has been well documented in tissue such as cerebrum (*5*, *6*) and retina (*7*) and has also been implicated in cell proliferation in T-lymphocytes (*8*) and melanoma cells (*9*). By comparison, activating protein-1 (AP-1), comprising a dimer of fos and jun subunits, preferentially binds to the phorbol 12-*O*-tetradecanoate-13-acetate-response element (TRE) (*10*). Regulation of AP-1 occurs at the level of gene transcription (*11*), while phosphorylation can modulate both its stability (*12*−*14*) and transcriptional activity (*15*). Like CREB, AP-1 plays a role in proliferation and differentiation of some cell types and may also be involved in apoptosis (*15*, *16*).

In order for TFs to regulate gene expression, they must gain access to the nucleus. Most proteins achieve this through the action of an intrinsic nuclear localization signal (NLS) which directs the transport of proteins through the nuclear envelope-localized nuclear pore complexes (NPCs) and into the nucleus. This process is mediated by a family of transporters or cytosolic receptor proteins known as importins (karyopherins) (*17*−*19*) which work in concert with the guanine nucleotide-binding protein Ran and regulatory proteins. In conventional NLS-dependent nuclear protein import, importin α (Impα) functions as an adaptor to link proteins containing a conventional NLS to importin β1 (Impβ) (*17*). The latter then mediates docking of the Impα-NLS-containing protein complex to the NPC, followed by energy-dependent translocation through the NPC (*20*). RanGTP dissociates the complex in the nucleus by binding to Impβ, and the individual importins are recycled back to the cytoplasm (*21*, *22*). Direct evidence suggests TFs localize in the nucleus through this NLS-dependent importin α/β-mediated nuclear transport pathway; however, this mechanism is essentially restricted to inducible TFs such as those of the nuclear factor NF-κB and STAT (signal transducer and activator of transcription) families (*23*−*25*).

Several proteins have recently been shown to be imported into the nucleus through pathways independent of Impα, which are mediated by a range of different Impβ homologues. In these pathways, the Impβ homologue recognizes the targeting signal of the transport substrate directly (*26*−*29*), as well as performing the NPC docking and Ran binding

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[1] Abbreviations: AP-1, activating protein-1; bZIP, basic leucine zipper; CREB, cAMP response element binding protein; CLSM, confocal laser scanning microscopy; DTAF, 5-(4,6-dichlorotriazinyl)-aminofluorescein; GFP, green fluorescent protein; IBB, importin β-binding; Imp, importin; NLS, nuclear localization signal; NPC, nuclear pore complex; PTHrP, parathyroid hormone-related protein; T-ag, Simian Virus SV40 large tumor antigen; TF, transcription factor; TRE, phorbol 12-*O*-tetradecanoate-12-acetate-response element.

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roles. The Impβ (Impβ1) homologues Impβ3 (Kap121p/Pse1p) (*30*, *31*), Impβ4 (Yrb4p) (*30*, *32*), and Sxm1 (Kap108p) (*33*), for example, have been shown to mediate the nuclear import of ribosomal proteins. In contrast, Impβ2 (transportin) appears to be largely responsible for the transport of hnRNPs in to and out of the nucleus (*28*, *34*, *35*), while Imp7 (RanBP7/Nmd5p/Kap119p) has been reported to mediate nuclear import of the yeast mitogen-activated protein kinase (MAPK) HOG1 (*36*) and general TF TFIIS (*37*). An analogous pathway has been described for proteins such as the yeast TF GAL4 (*38*), the related viral HIV-1 Rev (*39*) and HTLV-1 Rex (*40*) proteins, the T-cell protein tyrosine phosphatase (TCPTP) (*41*) and the polypeptide ligand parathyroid hormone-related protein (PTHrP) (*42*), where Impβ1 itself, independent of Impα, binds the nuclear import substrates directly. In the case of PTHrP, the ability of Impβ1 to function in nuclear import independently of Impα has been shown directly by reconstituting nuclear transport in vitro using purified, bacterially expressed components (*42*).

The present study examines the nuclear import pathway of the CREB and AP-1 TFs, demonstrating for the first time that CREB, AP-1, and its constituent fos and jun subunits are recognized by Impβ1 with high affinity and that Impβ1 can mediate docking of CREB to the nuclear envelope independent of Impα. Importantly, our quantitative approaches enable us to show that the affinity of interaction of Impβ with CREB is comparable to that of the conventional Impα/Impβ NLS-receptor for the well-characterized NLS of simian virus SV40 large tumor antigen (T-ag). We also show that RanGTPγS but not RanGDP is capable of dissociating the Impβ−CREB-GFP complex, and that disruption of the Ran gradient abolishes nuclear import. The results lend support for the existence of an Impβ1-specific and Ran-mediated nuclear import pathway specific for bZIP proteins, and possibly other classes of DNA-binding proteins. Constitutively nuclear TFs such as CREB thus utilize a nuclear import pathway distinct from that of inducible TFs.

MATERIALS AND METHODS

TF Expression, Purification, and Labeling. CREB was induced in exponentially growing *Escherichia coli* was as described (*43*); the cells were then resuspended in phosphate-buffered saline (PBS) containing 50 mM DTT and 50 mM EDTA (pH 8.0) before lysis by passage through a French press at 14 000 psi and heating to 75 °C for 10 min. The supernatant was separated from the insoluble fraction by centrifugation (15000*g*; 20 min) and applied to a P11 cellulose phosphate cation exchange column (Whatman) equilibrated in buffer A (10 mM HEPES pH 7.9, 0.5 mM EGTA, 1 mM $MgCl_2$, 5% glycerol, and 300 mM NaCl). CREB was eluted from the column with buffer A containing 1 M NaCl. Fractions were pooled and dialyzed against PBS and then aliquots stored at −70 °C prior to use. Fos and Jun, containing a 6-histidine tag at their N-terminus were expressed and purified by affinity chromatography using a Ni-NTA agarose column (Qiagen) as previously (*44*). Both were dialyzed against a final buffer containing 20 mM HEPES, pH 7.9, 500 mM NaCl, 10% glycerol, 1 mM DTT, and 0.1% NP40 and AP-1 reconstituted as described (*44*). Heterodimerization of fos and jun components was assessed by monitoring DNA binding affinity using native gel electrophoresis. Proteins were labeled with 5-(4,6-dichlorotriazinyl)aminofluorescein (DTAF; Molecular Probes) as described (*45*).

Expression and Purification of Importins and Ran. mImpα (PTAC58), mImpβ (PTAC97), yImpα (Kap60), and yImpβ (Kap95) importin subunits were expressed in *E. coli* as glutathione *S*-transferase (GST) fusion proteins and purified as described (*46*−*51*).

Ran-GST was expressed in *E. coli* as described (*52*) and bound to Glutathione Sepharose 4B (Amersham Pharmacia Biotech) beads. The GST fusion was then cleaved with thrombin (Amersham Pharmacia Biotech, 1 unit of thrombin/10 μg of Ran-GST) and free Ran eluted from the matrix. Thrombin was removed by incubation with Benzamidine Sepharose 6B (Amersham Pharmacia Biotech). Nucleotide loading of Ran was carried out as described (*53*); briefly, Ran (20 μM) was incubated for 30 min at room temperature in Ran loading buffer (50 mM Hepes, pH 7.4, 10 mM EDTA, 2.5 mM DTT, 1 mM ATP, and 1 mM GDP with or without 2.5 mM GTPγS) prior to loading onto Centricon 10 micro concentrator units (Millipore) to remove unbound nucleotides.

Construction, Expression, and Purification of CREB-GFP. pTRCAgfp was mutated to incorporate a single *Nhe*I site at the C-terminal end of the coding sequence of green fluorescent protein (GFP) using the primer 5′ GGATGAACTAGCTAGCTAACAG CTCGAGATCTGC 3′. The DNA-binding domain and NLS region of CREB (residues 271−342) were amplified by polymerase chain reaction (PCR) incorporating flanking *Nhe*I sites. The PCR product was then cloned into the vector and the fidelity of the construct verified by sequencing. For protein expression, *E. coli* strain BL21 (DE3) was grown at 28 °C to an OD of 0.6 whereby CREB-GFP expression was induced with 1 mM IPTG for 8 h at 28 °C. The bacteria were centrifuged and resuspended in buffer 1 (50 mM phosphate buffer, 10 mM Tris pH 8.0, 300 mM NaCl) containing lysozyme (5 mg/mL). Cell debris was removed by centrifugation and the supernatant loaded onto a Ni-NTA agarose (Qiagen) column. Prior to elution, nonspecifically bound proteins were removed from the column by washing the resin with 20 mM imidazole in buffer 1, after which CREB-GFP was eluted using 500 mM imidazole in buffer 1. The protein buffer was exchanged to PBS using a PD-10 column (Pharmacia) and aliquots stored at −70 °C.

In Vitro Nuclear Transport Assay and Kinetics. Nuclear import was analyzed at the single cell level using confocal laser scanning microscopy (CLSM) and mechanically perforated HTC cells, an approach completely comparable to detergent permeablised cells (*54*) in terms of the dependence of nuclear import on energy in the form of ATP and exogenous cytosol; this method has been used successfully to examine a number of importin α/β-recognized nuclear import substrates, including the *Drosophila* morphogen Dorsal (*25*), the tumor suppressor Rb (*47*), the *Xenopus* chromatin assembly component N1N2 (*52*), and Simian Virus SV40 large tumor antigen (T-ag) (*47*−*49*, *52*, *55*). Experiments were performed in a 5 μL volume containing cytosolic extract (untreated reticulocyte lysate, Promega), an ATP-regenerating system (0.125 mg/mL creatine kinase, 30 mM creatine phosphate, 2 mM ATP) and transport substrate

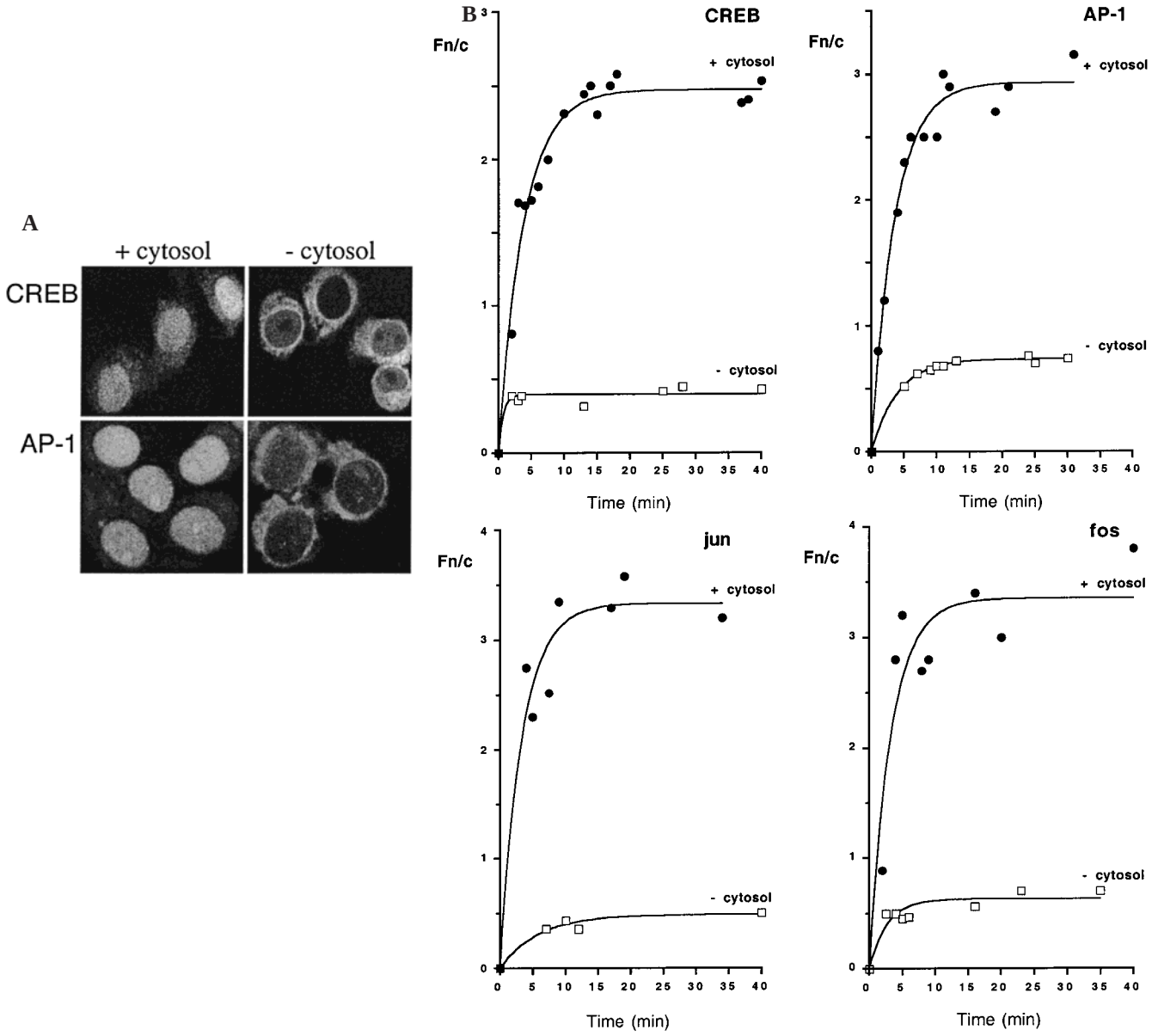

FIGURE 1: Dependence of in vitro nuclear import of CREB and AP-1 on cytosolic factors. Nuclear import was reconstituted in mechanically perforated HTC cells in the presence of an ATP regenerating system and in the absence or presence of exogenous cytosol as described in Materials and Methods. (A) CLSM images after 20 min. (B) Nuclear import kinetics from a single typical experiment performed as in panel A for CREB, AP-1, fos or jun. Each data point represents three to eight separate measurements for each of F_n (nuclear fluorescence), F_c (cytoplasmic fluorescence), and background fluorescence (see Materials and Methods), where the SD of the mean was not greater than 7% the value of the mean. Data were fitted for the function $F_n/c = F_n/c_{max}(1 - e^{-kt})$, where F_n/c_{max} is the maximal level of nuclear accumulation, k is the rate constant, and t is time in minutes. Pooled data are presented in Table 1.

(0.2 μM DTAF-labeled protein) or a control (70-kDa fluorescein isothiocyanate-labeled dextran; Sigma) to assess nuclear integrity. In inhibition experiments, cytosolic extract was preincubated at RT for 15 min with antibody (40 μg/mL) specific to Impα or Impβ (Santa Cruz Biotechnology), GTPγS at a final concentration of 200 μM, or RanGDP or RanGTPγS at a final concentration of 4 μM. In reconstitution experiments, perforated cells were rinsed two times in intracellular buffer to remove cytosolic components and importins were added at a final concentration of 1 μM. Image analysis and curve fitting was performed as described (*47*, *49*). As a control, a β-galactosidase fusion protein containing the importin α/β-recognized T-ag NLS was used (*55*).

ELISA-Based Binding Assay. An ELISA-based assay (*46*−*49*) was used to determine the affinity of binding between importin subunits and NLS-containing proteins. The latter were coated onto 96-well microtiter plates, incubated with increasing concentrations of importin subunits, and detection of bound importin-GST performed using a goat anti-GST primary antibody, an alkaline phosphatase-coupled rabbit anti-goat secondary antibody, and the substrate *p*-nitrophenyl phosphate (*46*). Absorbance measurements were performed over 90 min using a plate reader (Molecular Devices), with values corrected by subtracting absorbance both at 0 min and in wells incubated without importin (*46*).

Gel Mobility Shift Assay. Protein−protein interactions were assessed by gel mobility shift analysis whereby 500 nM of CREB-GFP was incubated with 25−3000 nM importins in the presence of binding buffer (10 mM Tris, 1 mM $MgCl_2$, 0.1% NP40, 10 mM DTT, 0.8 mM EDTA, 3% glycerol, and 1.5% sucrose) for 20 min. Samples were then subject to electrophoresis on a nondenaturing TBE polyacrylamide gel

and analyzed on the Fuji Film FLA3000 using the Image-Guage software.

Fluorescence Polarization Assay. Fluorescence anisotropy measurements (*56*) of CREB-GFP (see also *57*) were carried out using an SLM 8100 photocounting spectrophotometer fitted with Glan-Thompson polarizes. Excitation was at 490 nM and light emitted from the fluorophore was collected after passage through a 533 nm cutoff filter. For each assay, CREB-GFP was diluted to a final concentration of 30 nM in PBS, and anisotropy changes [calculated according to Weber (*56*); $A = (I_{||} - I_{\perp})/(I_{||} + 2I_{\perp})$, where A is anisotropy, and $I_{||}$ and $I_{\perp}$ are the parallel and perpendicular emitted light intensity, respectively] monitored as aliquots of importin and Ran were successively added.

RESULTS

The basic DNA binding domains of CREB and AP-1 have been implicated as possessing NLS function (*58*−*62*), CREB residues 301−309 (RRKKKEYVK) in particular having been shown to be both necessary and sufficient for nuclear targeting; mutation of lysine 304 markedly diminishes activity (*61*). Since nothing is known regarding the specific pathway by which CREB and AP-1 gain access to the nucleus, however, we set out to determine the respective nuclear import mechanism(s).

CREB and AP-1 Nuclear Import Is Dependent on Cytosolic Factors. CREB and the fos and jun components of AP-1 were bacterially expressed, purified, and confirmed for specific DNA binding activity in gel mobility shift assays. The proteins were then labeled with DTAF, and nuclear import kinetics assessed using an in vitro nuclear transport assay in conjunction with CLSM (*54*). CREB and AP-1 were found to accumulate within the nucleus in the presence of cytosolic factors and an ATP regenerating system (Figure 1A). CREB and AP-1 exhibited levels of maximal nuclear accumulation (F_n/c_{max}) 2.5- and 3- fold higher than those in the cytoplasm, respectively (Figure 1B). Half-maximal ($t_{1/2}$) nuclear accumulation of CREB and AP-1 was attained within 2.7 and 2.6 min, respectively (Table 1). The component subunits of AP-1, fos and jun, were also found to accumulate in the nucleus, with F_n/c_{max} values of 3.1 and 3.2, respectively (Figure 1B, Table 1). To test whether nuclear import was dependent on cytosol, transport assays were also performed in the absence of cytosolic extract. Nuclear accumulation was abolished (Figure 1A) with the F_n/c_{max} values reduced to 0.4 and 0.74 for CREB and AP-1 respectively (Figure 1B, Table 1), indicative of exclusion from the nucleus. Similar results were obtained for fos and jun (Figure 1B, Table 1, and data not shown).

CREB and AP-1 Are Recognized by Importin β with High Affinity. Since the nuclear import of CREB and AP-1 was dependent on exogenous cytosol, we decided to test whether CREB and AP-1, as well as jun and fos, were recognized by Impα or Impβ. Mouse and yeast Impα and Impβ were expressed as GST-fusion proteins, and interactions with CREB and AP-1 quantified using an ELISA-based binding assay, previously used to determine the binding affinities (apparent dissociation constants, K_d) of importin subunits for a variety of different NLS-containing proteins (*46*−*49*). We found that mImpβ recognized CREB with 20-fold higher affinity than mImpα; the yeast equivalents showed a similar trend, with yImpβ showing a 6-fold higher affinity than yImpα (Figure 2A, Table 2). The fact that the results for Impβ alone corresponded well to those for the Impα/β heterodimer suggested that the interaction of the latter is mediated through Impβ. AP-1 was also recognized by Impβ with higher affinity than Impα in the case of both mouse and yeast homologues (Figure 2B), with a K_d of 45 nM for mImpβ as compared to 140 nM for mImpα (Table 2). In the case of both CREB and AP-1, mImpα and mImpβ bound with higher affinity than their yeast counterparts, indicative of the closer relatedness in evolution of rat/human to mouse than to yeast.

Table 1: Kinetics of Nuclear Import of BZIP TFs and T-ag-NLS-Containing Control in Vitro

		nuclear import parameter[b]		
protein	addition[a]	F_n/c_{max}	$t_{1/2}$ (min)	n
CREB	+cytosol[c]	2.5 ± 0.1	2.7 ± 0.9	(4)
	−cytosol	0.40 ± 0.08	ND[d]	(3)
	+cytosol + anti-Impα	2.0 ± 0.2	1.8 ± 0.7	(4)
	+cytosol + anti-Impβ[c]	0.52 ± 0.02	ND[d]	(3)
	+cytosol + GTPγS[c]	1.5 ± 0.1	2.1 ± 0.5	(3)
	+cytosol + RanGDP	2.5 ± 0.2	2.6 ± 0.5	(3)
	+cytosol + RanGTPγS[c]	0.98 ± 0.07	ND[d]	(2)
AP-1	+cytosol	3.0 ± 0.1	2.6 ± 0.7	(4)
	−cytosol	0.74 ± 0.06	ND[d]	(3)
Fos	+cytosol	3.1 ± 0.3	2.9 ± 0.6	(2)
	−cytosol	0.5 ± 0.1	ND[d]	(2)
Jun	+cytosol	3.2 ± 0.2	3.2 ± 0.9	(2)
	−cytosol	0.5 ± 0.1	ND[d]	(2)
T-ag-NLS-βGal	+cytosol[e]	2.7 ± 0.2	4.4 ± 0.6	(2)
	+cytosol + anti-Impα[e]	1.01 ± 0.03	ND[d]	(2)
	+cytosol + anti-Impβ[e]	1.34 ± 0.07	ND[d]	(2)

[a] All additions include an ATP regenerating system. [b] Results represent the mean ± SE from data (see Figures 1B and 5B) fitted to the function $F_n/c = F_n/c_{max}(1 - e^{-kt})$, where F_n/c_{max} is the maximal level of nuclear accumulation, k is the rate constant, and t is time in minutes. [c] Significant differences were observed between F_n/c max values for CREB with and without anti-Impβ antibody ($p < 0.0001$), and with and without GTPγS ($p = 0.001$) and RanGTPγS ($p = 0.0006$). [d] ND, not able to be determined due to low nuclear accumulation/nuclear exclusion. [e] Significant differences were observed between F_n/c max values for T-ag-NLS-β-Gal in the absence and presence of anti-Impα ($p = 0.014$) and anti-Impβ ($p = 0.0234$) antibodies.

Fos and jun showed a similar trend to AP-1, with Impβ binding with higher affinity than Impα (Figure 2C, Table 2). Results implied that the high affinity mImpα/β heterodimer binding to AP-1 is mediated predominantly through jun, since the latter showed significantly higher maximal binding and a lower K_d than Fos. Thus, while both jun and fos are clearly able to localize in the nucleus in the absence of the other subunit (see Figure 1B, bottom panels), transport of AP-1 may be mediated predominantly through Impβ binding to jun.

To confirm the direct recognition by Impβ in the case of CREB, a plasmid expressing a GFP fusion protein containing the NLS and DNA-binding domain of CREB (residues 271−342) was constructed, and the protein expressed and purified (see Materials and Methods). The ability of Impα and Impβ to bind CREB-GFP was initially assessed by native gel electrophoresis (Figure 3A) followed by fluorescence imaging. Impβ bound CREB-GFP to a greater extent than Impα as indicated by the low percent of unbound (nonshifted) CREB-GFP (Figure 3B, left panel) and the high percent of

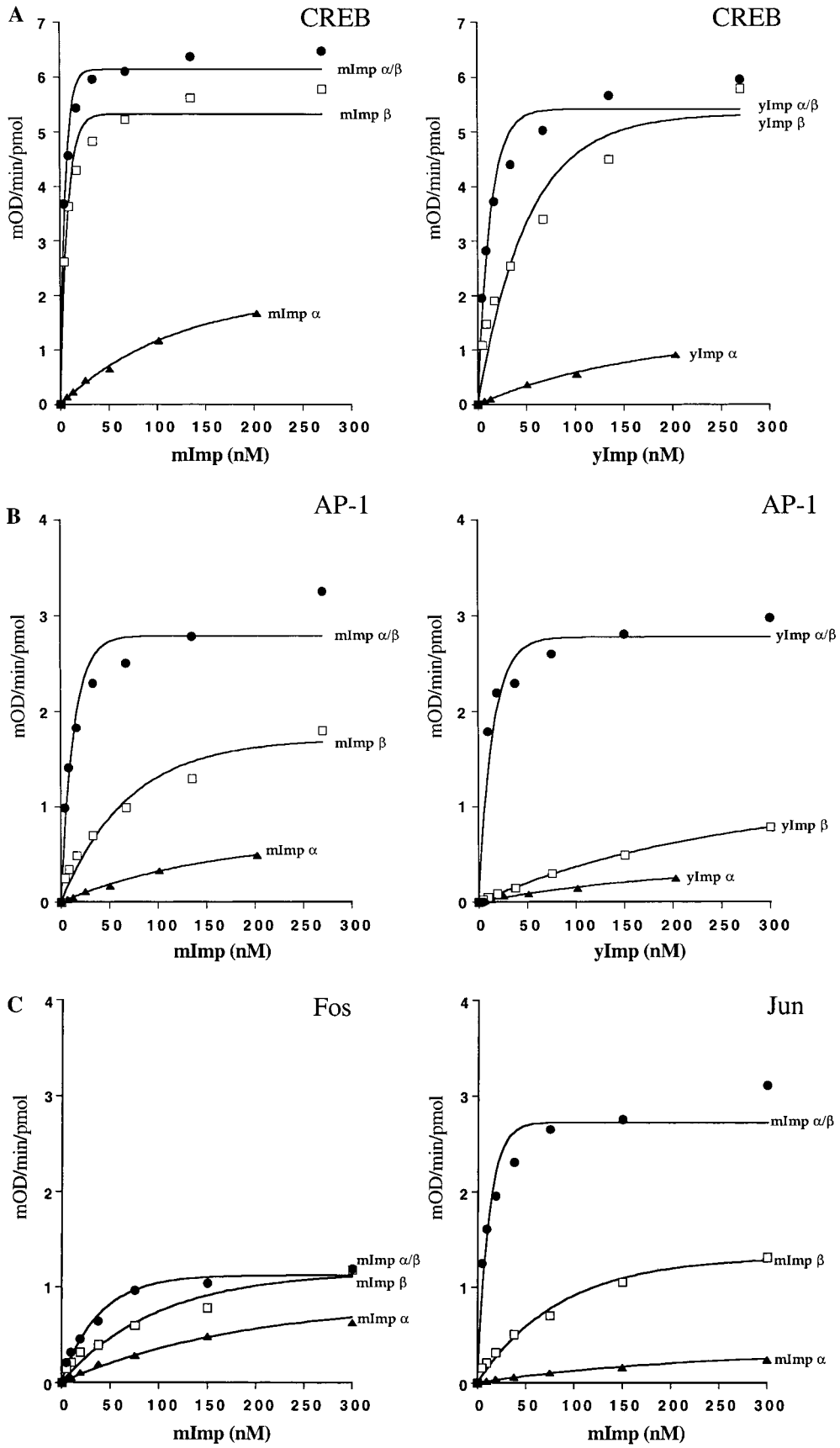

FIGURE 2: Importin β recognizes CREB and AP-1 with higher affinity than Impα as quantitated using an ELISA-based binding assay. Microtiter plates were coated with CREB (A), AP-1 (B), fos, or jun (C) as indicated and incubated with increasing concentrations of mouse or yeast importin subunits. Data were fitted for the function $B(x) = B_{max}(1 - e^{-kx})$, where x is the concentration of importin, and B is the level of importin bound, with the apparent dissociation constants (K_d values, representing the importin concentration yielding half-maximal binding) indicated. The results are from a single typical experiment performed in triplicate with pooled data shown in Table 2.

Table 2: Binding Parameters of CREB, AP-1, Fos, and Jun for Importins as Determined Using an ELISA-Based Binding Assay

	mouse importins[a]									yeast importins[a]								
	mImpα			mImpβ			mImpα/β			yImpα			yImpβ			yImpα/β		
	K_d	B_{max}	n	K_d	B_{max}	n	K_d	B_{max}	n	K_d	B_{max}	n	K_d	B_{max}	n	K_d	B_{max}	n
CREB	120 ± 20	35 ± 7	5	6 ± 2	87 ± 14	5	4 ± 1	100 ± 13	5	245 ± 10	23 ± 10	3	38 ± 4	86 ± 5	3	8 ± 2	88 ± 15	3
AP-1	140 ± 20	25 ± 4	3	45 ± 5	66 ± 16	3	8 ± 2	100 ± 18	3	300 ± 20	14 ± 4	3	200 ± 25	40 ± 16	3	9 ± 3	99 ± 22	3
fos	150 ± 20	36 ± 15	4	65 ± 10	44 ± 18	4	30 ± 5	46 ± 7	4									
jun	160 ± 15	9 ± 2	4	70 ± 10	44 ± 11	4	10 ± 4	92 ± 9	4									

[a] Data represent the mean ±SE for the apparent dissociation constant (K_d) and maximal binding, determined (see Figure 2) as outlined under Materials and Methods.

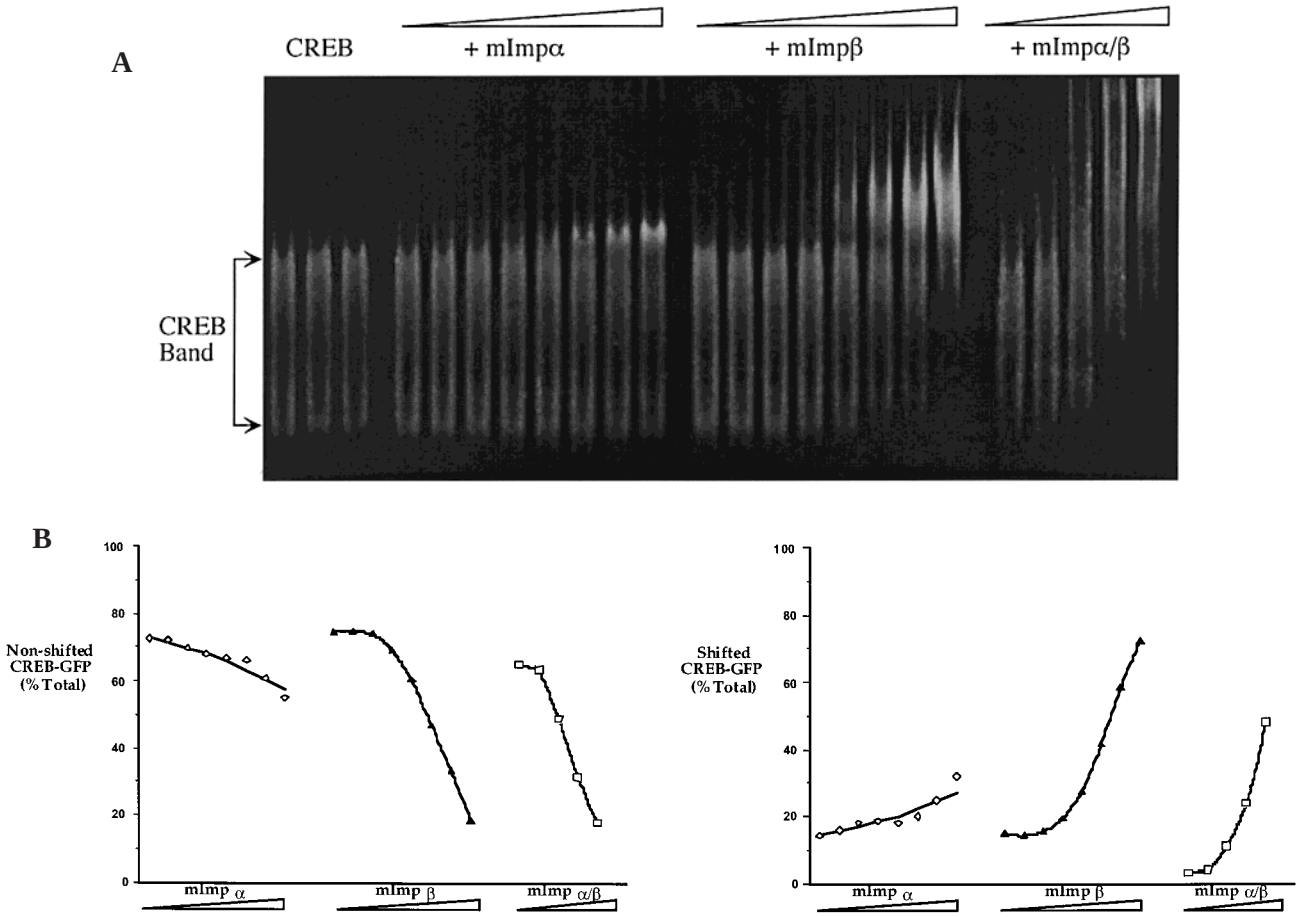

FIGURE 3: Impβ recognizes CREB with higher affinity than Impα as shown by native gel electrophoresis. (A) Fluorescent image of a 5% native polyacrylamide gel after electrophoresis for 2 h at 30 mA. The position of CREB-GFP is shown in the absence (three left lanes) or presence of preincubation with the mouse importin subunits indicated (25−3000 nM) prior to electrophoresis. (B) Results for quantitation of fluorescence (ImageGauge software) from panel A are shown; in the left panel, results are shown for the relative amount of unshifted CREB-GFP (main band, indicated by arrows) as a percentage of the total fluorescence; in the right panel, the relative amount of CREB shifted due to importin binding is expressed as a percentage of the total fluorescence.

lower mobility (shifted) CREB-GFP due to complexation with the importin (Figure 3B, right panel). The estimated affinity of binding for Impβ to CREB-GFP based on the electrophoretic results indicated a greater K_d value (indicative of lower binding affinity) than that estimated using the ELISA assay. This was attributed to dissociation of the protein−protein complex during electrophoretic movement through the gel, which has been well documented for other proteins (*63*).

Binding of CREB-GFP by importins was further assessed by fluorescence polarization, which allows complexation interactions to be examined in solution. Binding of importins to CREB-GFP was measured as a change in anisotropy, which relates to the state of complexation of the fluorescent molecule. Consistent with ELISA and gel-mobility shift analysis, CREB-GFP was bound in solution by Impβ with higher affinity (lower K_d) than Impα (Figure 4A). The estimated affinity of binding (K_d 35 nM) was comparable to that determined in the ELISA.

Thus, based on the results from a range of assays, CREB is clearly recognized by Impβ with higher affinity than Impα. This high affinity recognition of CREB and AP-1 by Impβ contrasts strongly with our observations for the conventional NLS-containing T-ag (*46*), Rb tumor suppressor (*47*), *Xenopus laevis* chromatin assembly factor N1N2 (*52*, *64*), and *Drosophila* TF Dorsal (*25*), all of which contain NLSs recognized by Impα and not directly by Impβ. These observations are thus consistent with the idea that the CREB

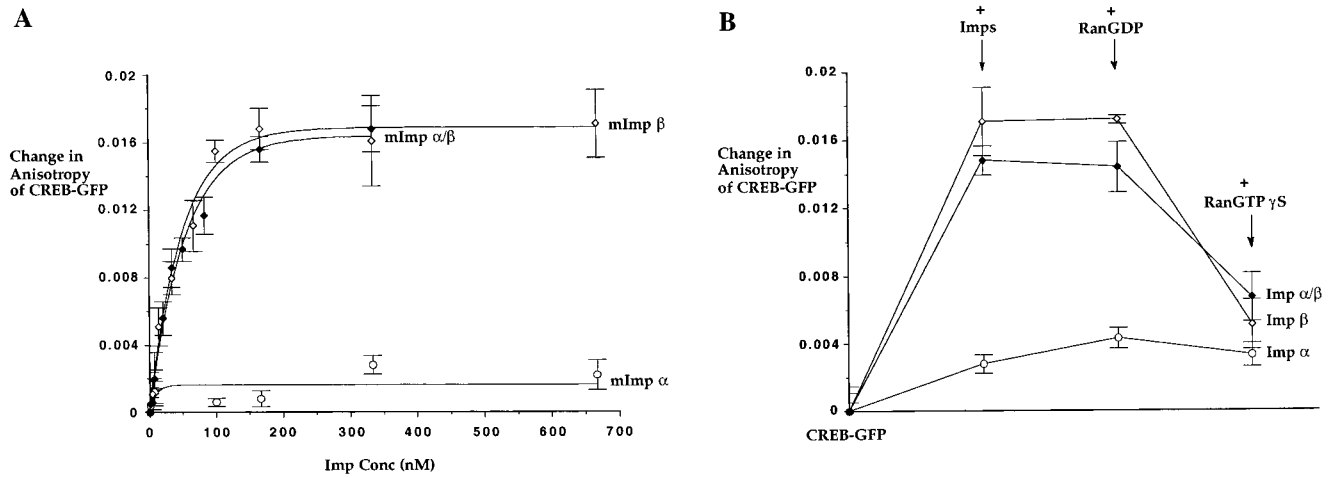

FIGURE 4: Impβ binds CREB-GFP with higher affinity than Impα (A) and the complex can be dissociated by RanGTP (B) as determined by fluorescence polarization. (A) Fluorescence polarization measurements for CREB-GFP in the presence of increasing concentrations of mImpα, mImpβ and mImpα/β. Data points represent the average of 3 polarization measurements, performed in triplicate, with the SD indicated. Curves were fitted for the function $A(x) = A_{max}(1 - e^{-kx})$, where x is the concentration of importin, and A is the anisotropy change. (B) Anisotropy changes of CREB-GFP showing the addition of mImps at saturating levels (333 nM) followed by RanGDP or RanGTPγS (133 nM) as indicated.

and AP-1 TFs may be transported to the nucleus through a distinct Impβ-mediated pathway.

Nuclear Transport of CREB requires Impβ and Ran. To test whether CREB nuclear import may be independent of Impα, cytosol was pretreated with antibodies specific for either Impα or Impβ, and the kinetics of CREB nuclear import assessed in vitro. Anti-Impβ antibodies abolished nuclear transport completely while anti-Impα antibodies did not reduce nuclear accumulation significantly (Figure 5). By comparison, both the anti-Impα and anti-Impβ antibodies inhibited nuclear accumulation of the Impα/Impβ-recognized NLS-containing T-ag-NLS-β-galactosidase control protein significantly ($p < 0.015$ and $p < 0.025$ respectively; Table 1). The results indicated that the nuclear import pathway for CREB was dependent on Impβ, and independent of Impα.

The nature of the guanine nucleotide bound by Ran (*65*, *66*) determines the directionality of nuclear transport (*67*, *68*); in the nucleus, Ran is maintained predominantly in its GTP form by the nucleotide exchange factor RCC1, whereas RanGDP is constantly generated in the cytoplasm by the GTPase activating protein RanGAP1. It has previously been shown that disruption of the RanGTP gradient across the nuclear envelope through the use of nonhydrolyzable analogues of GTP such as GTPγS can inhibit nuclear import (*66*), so we decided to test whether CREB nuclear import could be inhibited by GTPγS. The F_n/c_{max} was significantly ($p < 0.002$) reduced to a value of 1.5 (Figure 5, Table 1). To demonstrate formally that this was attributable to disruption of the Ran gradient, we assessed the effect on transport of adding Ran preloaded either with GDP or GTPγS. We found that the addition of RanGTPγS to the cytosol significantly reduced nuclear accumulation (F_n/c_{max} of 0.98) while the addition of RanGDP did not significantly reduce nuclear transport (Figure 5; Table 1). Clearly, Impβ-mediated nuclear import pathway is modulated by Ran according to the guanine nucleotide bound.

Importin β Mediates Docking of CREB at the NPC; Ran Mediates Impβ-CREB Release. As a step toward reconstituting the nuclear import pathway of CREB in vitro, we attempted to reproduce the docking step at the nuclear envelope using purified transport components. In the presence of Impβ, Impα/β, but not Impα alone, CREB localized strongly at the nuclear envelope (Figure 6), indicating that Impβ is necessary and sufficient for docking of CREB at the nuclear envelope; Impα is not required. RanGTPγS but not RanGDP reduced the accumulation of CREB at the nuclear envelope (Figure 6), consistent with the idea that RanGTP is able to dissociate the CREB−Impβ complex. Image analysis using the line plot mode (*45*) indicated c. two-fold higher levels of CREB at the nuclear envelope than in the cytoplasm in the presence of either Impβ or Imp α/β. Impα alone, in contrast, did not effect a significant increase in CREB nuclear envelope association above that in its absence. In the presence of RanGDP, Impβ-mediated nuclear envelope association of CREB was reduced only marginally, in contrast to the significant reduction ($p < 0.005$) effected by RanGTPγS; RanGTPγS thus can prevent Impβ-mediated binding of CREB to the nuclear envelope.

The ability of RanGTP to dissociate the CREB−Impβ complex was confirmed using fluorescence polarization for the first time, whereby Impβ binding to CREB-GFP was initially saturated, and then RanGDP and RanGTPγS successively added (see Figure 4B). Whereas RanGDP did not affect binding of Impβ to CREB-GFP, RanGTPγS dissociated the Impβ−CREB-GFP complex, effecting a marked reduction in anisotropy. These results were thus consistent with Ran's role in CREB nuclear import being to effect release from Impβ within the nucleus after transport through the NPC.

DISCUSSION

The present study represents the first quantitative analysis examining the nuclear import pathways of the TFs CREB, AP-1, and jun and fos. We show that Impβ binds CREB and AP-1 with significantly higher affinity than Impα, and using specific antibodies demonstrates the requirement of Impβ but not Impα for CREB nuclear import, in contrast to transport conferred by Impα/β-recognized NLSs. Furthermore, we demonstrate that Impβ is necessary and sufficient to mediate docking of CREB to the nuclear envelope. Our

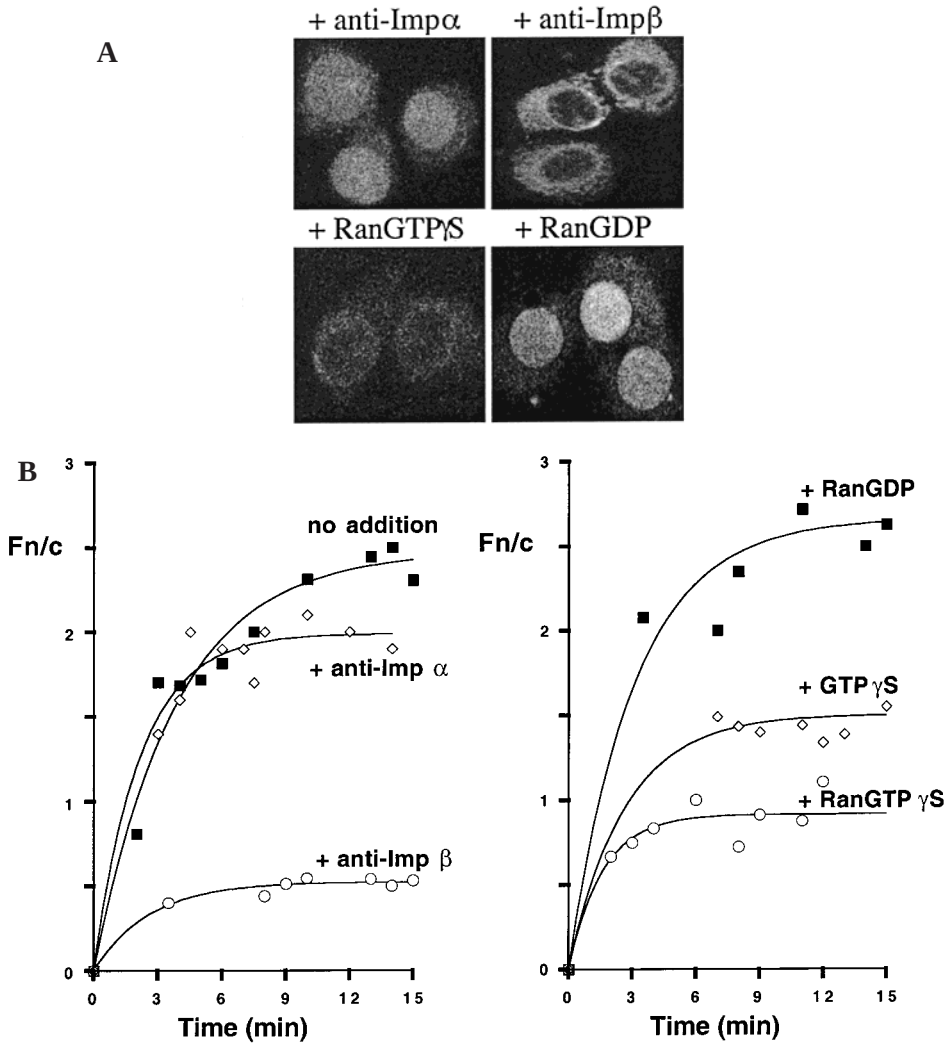

FIGURE 5: Nuclear accumulation of CREB requires Impβ and RanGDP. Nuclear import was reconstituted in mechanically perforated HTC cells as described in the legend to Figure 1 in the presence of an ATP-regenerating system with cytosol alone (no addition), or pretreated with antibodies for Impα or Impβ (40 μg/mL), with GTPγS (200 μM), RanGTPγS (4 μM), or RanGDP (4 μM) (A) CLSM images after 10 min in the presence of anti-Impα, anti-Impβ, RanGTPγS, and RanGDP. (B) Nuclear import kinetics from a single typical experiment. Each data point represents four to eight separate measurements for each of F_n, F_c, and background fluorescence (see Materials and Methods), where the SD was not greater than 10% of the value of the mean. Pooled data are presented in Table 1.

results imply the existence of a pathway, distinct from that conferred by conventional NLSs such as that of T-ag where Impα is the NLS receptor and Impβ mediates docking at the NPC. Importantly, our quantitative approaches demonstrate that the affinity of recognition of CREB by Impβ (nM) is completely comparable to that of conventional NLSs such as those of T-ag and N1N2 (*52*) by Impα/β. This indicates that the initial step of target signal recognition in the case of CREB and AP-1 is likely to be equally efficient to that of T-ag, N1N2 and other transcription factors. Since target signal recognition is critical in determining the efficiency of nuclear transport (*29*), the clear and important implication is that the Impβ-mediated nuclear import pathway is likely to be comparable in efficiency to that mediated by Impα/β.

Our study indicates that Ran is an important component in the Impβ-mediated transport pathway for CREB and presumably AP-1. Using fluorescent polarization assays for the first time, we show that RanGTPγS, in contrast to RanGDP, is able to dissociate the Impβ−CREB complex, as well as inhibit the accumulation of CREB in the nucleus. These results are consistent with the idea that Ran's role in CREB nuclear import is at the nucleoplasmic release step, after transport through the NPC (*22*, *66*, *68*). We speculate that the Impβ- and Ran-modulated nuclear import pathway utilized by CREB may apply to constitutively nuclear DNA-binding proteins in general, consistent with our previous observations that GAL4 is recognized by Impβ and not Impα (*38*). Inducible TFs such as STAT1 and NF-κB family members would appear to be imported into the nucleus through the more conventional Impα-dependent pathway (*23*−*25*).

In addition to its ability to mediate the nuclear import of DNA-binding proteins and other import substrates, as indicated by the study here and elsewhere, Impβ possesses Ran-, Impα-, and nucleoporin-binding activities (*69*, *70*). In preliminary experiments using fluorescence polarization (not shown), we have observed that the Impβ interaction with CREB most likely occurs within the C-terminal region of Impβ, thus, not colocalizing to the region interacting with Ran which maps to the Impβ N-terminal region (*69*−*71*). The crystal structure of Impβ indicates that the "NLS like" importin β-binding (IBB) domain of Impα interacts with residues within HEAT repeats 7−19 (*72*), and it has been suggested that proteins recognized directly by Impβ may do

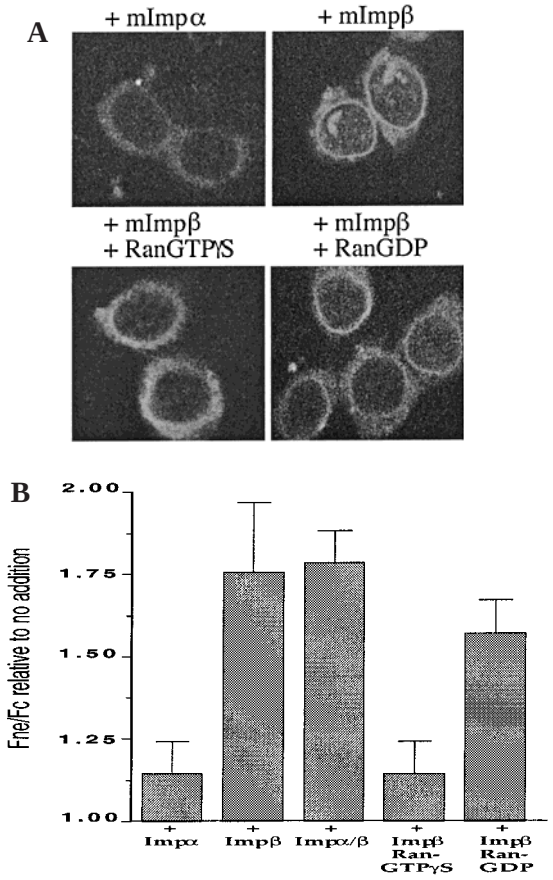

FIGURE 6: Impβ is necessary and sufficient for docking CREB at the NPC, while RanGTPγS prevents recognition of CREB. Docking at the nuclear envelope was reconstituted in mechanically perforated HTC cells in the absence of cytosol, in the absence or presence of bacterially expressed nuclear transport factors Impα and/or Impβ (1 μM), and/or RanGTPγS or RanGDP (4 μM). (A) CLSM images after 10 min in the presence of Impα, Impβ, Impβ and RanGDP, and Impβ and RanGTPγS. (B) Quantitation of docking at the nuclear envelope relative to cytoplasmic fluorescence (F_{ne}/F_c) using the NIH Image 1.60 analysis software (line plot mode) (*45*). Results are the mean ± SEM for a minimum of five determinations of each of F_{ne} (nuclear envelope-associated fluorescence), F_c, and background fluorescence (see Materials and Methods).

so through homology to the Impα IBB domain (*72*). Our results indicate that it is unlikely for CREB to bind the same site on Impβ as the IBB domain, since our results show that the Impα/β heterodimer binds CREB with as high affinity as Impβ, implying that (a) the region of Impβ involved in binding Impα is not involved in CREB recognition and (b) the heterodimer binds through Impβ (Figures 2A, 3A, and 4A). Accordingly, we propose that CREB does not make direct contact with Impβ through sites important for recognition of the Impα IBB domain, but rather, a distinct region within the C-terminus of Impβ may be involved in recognizing nuclear import substrates. Consistent with this idea is the fact that the TCPTP NLS can be recognized by truncated Impβ that is unable to bind Impα (*41*), and that the region of Impβ recognizing the PTHrP NLS (*42*, and unpublished results) is distinct from that binding the IBB domain (*72*).

Apart from GAL4 and bZIP TFs, other proteins imported to the nucleus through Impβ, without involvement of Impα, include TCPTP (*41*), PTHrP (*42*), cyclin B1 (*73*), and Rex (*40*) and Rev (*39*). This indicates that Impβ-mediated nuclear import is not exclusive to DNA-binding proteins. In addition, it is clear that not all TFs localize in the nucleus through Impβ; known nuclear import pathways for TFs include mediation of the transport of the TATA-binding protein by Kap114p (*74*), of the general TFs TFIIS and TFIIA by Kap119p (*37*) and Kap122p (*75*), respectively, and of the yeast TF Pho4 by Impβ3 (Pse1p) (*76*, *77*). Yet other TFs, and in particular inducible TFs such as those of the NF-κB family (*23*, *25*) and STAT1 (*24*), would appear to be imported into the nucleus through the conventional importin α/β1-mediated pathway. The cell thus possesses multiple nuclear import pathways for different types of TF enabling fine control to be exerted over individual classes of TFs (*18*, *29*). Known mechanisms for regulating nuclear protein import include preventing recognition of the nuclear targeting signal through intramolecular or intermolecular masking, and enhancement of nuclear targeting signal recognition by phosphorylation which influences the initial interaction of the targeting signal and importin (*26*, *29*, *78*). Significantly, the latter appears to relate largely to inducible TFs which, as indicated, are importin α-recognized (*23*−*25*). It would thus appear that TFs constitutively required in the nucleus such as CREB or general TFs are imported through pathways independent of both Impα, and the accompanying very specific regulatory mechanisms through phosphorylation, etc. Through multiple importins, and regulation of nuclear targeting signal recognition, the cell is able to regulate the import of specific classes of proteins selectively (*26*, *29*).

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