Nucleolar Protein B23 Stimulates Nuclear Import of the HIV-1 Rev Protein and NLS-Conjugated Albumin†

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ABSTRACT: Nucleolar phosphoprotein B23 is a putative ribosome assembly factor with a relatively high affinity for peptides containing sequences of nuclear localization signals (NLSs) of the SV40 T-antigen type [Szebeni, A., Herrera, J. E., & Olson, O. J. (1995) *Biochemistry 34*, 8037−8042]. The effects of protein B23 on nuclear import were determined by an *in vitro* assay [Dean, D. A., & Kasamatsu, H. (1994) *J. Biol. Chem. 269*, 4910−4916] using NLS peptide-conjugated bovine serum albumin (NLS−BSA) or the HIV-1 Rev protein as substrates for import into isolated rat liver nuclei. The import was ATP-dependent and inhibited by wheat germ agglutinin or by an antibody against p97, a component of the nuclear import system. The rate of import of either substrate was increased if protein B23 was added to the incubation medium. Similar enhancements of import were seen with both isoforms (B23.1 and B23.2). The stimulatory effect on Rev protein import was saturable with maximum stimulation (2−3-fold) at a molar ratio of protein B23:Rev of approximately 1:1. Phosphorylation of protein B23.1 by casein kinase II produced an additional doubling of the import rate. This effect was not seen if protein B23.1 was phosphorylated with a cdc2 type protein kinase. Mutant forms of protein B23.1 in which the nuclear localization signal was either deleted or altered did not stimulate import of the substrates. These results suggest that protein B23 plays a role as an accessory factor in the nuclear import of the NLS-containing proteins and that phosphorylation at sites in the highly acidic segments of the protein enhances the stimulatory effect.

In eukaryotic cells, the nuclear envelope serves as a semipermeable barrier to separate the processes of translation and transcription. Bidirectional traffic between the cytoplasm and the nucleus takes place through nuclear pores which span the inner and outer layers of the nuclear envelope (Feldherr, 1992). Proteins that function in the nucleus such as histones, transcriptional regulatory proteins, DNA and RNA polymerases and ribosomal proteins are selectively imported into the nucleus from the cytoplasm. At the same time, tRNAs, mRNAs, snRNAs, and ribosomal subunits are exported to the cytoplasm. In addition, certain proteins have been shown to shuttle between the two cellular compartments (Borer et al., 1989; Meier & Blobel, 1990; Mandell & Feldherr, 1990; Piñol-Roma & Dreyfuss, 1992).

The nuclear pore complex (NPC)[1] is a large and symmetrical supramolecular gate (Akey, 1989; Akey & Goldfarb, 1989). Ions, metabolites, and small proteins can enter the nucleus by passive diffusion through the NPC which has an open channel with a diameter of 10 nm. However, most proteins and RNAs over 20−40 kDa are too large to diffuse through this channel [Paine et al., 1975; Peters, 1987; Nigg et al., 1991; Forbes, 1992; Gerace, 1992; for review see Görlich and Mattaj (1996) and Csermely et al. (1995)]. The nuclear accumulation of proteins via the nuclear pore complex is directed by relatively short basic sequences called nuclear localization signals (NLS) contained in these proteins (Feldherr, 1992; Dingwall & Laskey, 1991; Boulikas, 1994; Fabre & Hurt, 1994). Two major classes of NLS sequences have been identified. The most studied is the NLS found in the prototypical SV40 large T antigen (PKKKRKV) in which five of the seven amino acid residues are basic (Kalderon et al., 1984). The second class of NLS is typified by that found in the *Xenopus* protein nucleoplasmin (Dingwall et al., 1988; Robbins et al., 1991) where two critical basic domains are separated by a spacer of ten noncritical amino acid residues.

The import process can be separated into two distinct steps. The first is the ATP-independent docking of NLS-containing protein to the nuclear pore complex which is followed by a slower ATP- and temperature-dependent translocation through the pore (Richardson et al., 1988; Newmeyer & Forbes, 1988; Moore & Blobel, 1994). Both steps appear to require multiple cytosolic factors. The binding step involves at least two kinds of proteins, a receptor of M_r 54/56 kDa and a 97 kDa protein that binds with the receptor to the nuclear pore complex (Adam & Adam, 1994; Chi et al., 1995). These proteins probably correspond to importin 60 and 90 (Görlich et al., 1995) or karyopherin α and β (Moroianu et al., 1995), respectively. The translocation step involves a small GTP-

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[1] Abbreviations: NPC, nuclear pore complex; NLS, nuclear localization signal; cdc, cell division cycle; CKII, casein kinase II; HIV, human immunodeficiency virus; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; ConA, concanavalin A; WGA, wheat germ agglutinin; DTAF, dichlorotriazinylaminofluorescein.

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binding protein called Ran/TC4 (Moore & Blobel, 1994; Melchior et al., 1993).

Although NLS sequences have been found in a large number of proteins, only a few proteins with which they potentially interact have been identified. One of these is nucleolar protein B23, which has been shown to bind peptides containing NLS sequences from the SV40 T antigen (Goldfarb, 1988) and to specifically interact with these in a 1:1 stoichiometry (Szebeni et al., 1995). Protein B23 (also called nucleophosmin, numatrin, or NO38) is an abundant multifunctional phosphoprotein predominantly located in the nucleolus. This protein has been shown to shuttle between the nucleus and the cytoplasm (Borer et al., 1989). Furthermore, protein B23 forms a specific complex with the human immunodeficiency virus-1 (HIV-1) Rev protein (Fankhauser et al., 1991; Umekawa et al., 1993) which is also localized to the nucleoli of HIV-infected cells (Cullen et al., 1988). The highly acidic segments of protein B23 (Mamrack et al., 1977) appear to be responsible for binding the nuclear localization signal sequences (Adachi et al., 1993). It has been suggested that these segments of protein B23 are also capable of binding basic sequences in ribosomal proteins which are transient components of the nucleolus during their assembly into ribosomes (Olson, 1990). Taken together, the above data suggest that protein B23 plays a role in the import of proteins into the nucleus, especially those proteins whose destination is the nucleolus.

Protein B23 exists in cells as a phosphoprotein. Phosphorylation has been shown at one or more typical casein kinase II (CKII) sites (Chan et al., 1986) and also at cdc2 type kinase sites (Jones et al., 1981; Peter et al., 1990; Feuerstein & Randazzo, 1991). The former sites are phosphorylated during interphase while the latter sites are phosphorylated only during metaphase (Peter et al., 1990). The phosphorylation of protein B23 at the cdc2 type sites is believed to be related to the breakdown of the nucleolus during mitosis, whereas CKII phosphorylation has been proposed to play a role in ribosome biogenesis (Olson, 1990). Interestingly, recent studies showed that phosphorylation of protein B23 by CKII increased its affinity for peptides based on NLS sequences, while phosphorylation by a cdc2 type kinase had no effect on NLS peptide binding (Szebeni et al., 1995). This suggests that CKII phosphorylation may be involved at the level of nuclear import.

Although the proteins of the nuclear import system are rapidly becoming characterized, it is possible that accessory factors also participate in the import process. The characteristics of protein B23 described above suggest such a role at some stage of nuclear import. The current work was initiated to extend previous studies on the binding of NLS peptides to protein B23 (Szebeni et al., 1995) by asking whether protein B23 is able to alter the rates of nuclear import of NLS-containing substrates. The substrates chosen for this were bovine serum albumin to which peptides containing the SV40 T-antigen were attached and the HIV-1 Rev protein, which localizes to the nucleolus and has a highly basic NLS. It was found that the presence of protein B23 stimulates the rate of import of both substrates into isolated rat liver nuclei. This stimulation was further enhanced if protein B23 was phosphorylated by casein kinase II.

EXPERIMENTAL PROCEDURES

Protein Preparations. Recombinant proteins B23.1 and B23.2 used in these studies were produced in *Escherichia coli* and purified essentially as previously described (Umekawa et al., 1993) except that the respective cDNAs were inserted into the pET 11c vector for expression (Novagen). Recombinant protein B23.1ΔNLS was obtained from a mutant in which the NLS region was deleted by using the PCR SOEing technique (splicing by overlap extension). Mutant protein B23.1 QNLS was prepared by a similar method, except that lysines 154, 155, and 157 were substituted with glutamines. The mutant proteins were expressed in *E. coli* BL21-DE3 after cloning into pET15b and were purified by nickel chelate and affinity chromatography. Recombinant Rev protein was expressed and purified as previously described (Wingfield et al., 1991). The monoclonal antibody to p97 was prepared as previously described (Chi et al., 1995).

Protein Phosphorylation. Recombinant protein B23 was phosphorylated in vitro with either casein kinase II (Boehringer-Mannheim) or the cdc2 kinase (Upstate Biotechnology) under the reaction conditions described below. For casein kinase II phosphorylation, samples of protein B23.1 or B23.2 (0.3 mg/mL) were incubated in a reaction mixture containing 1 milliunit/mL of casein kinase II, 20 mM MES buffer (pH 7.2), 130 mM KCl, 0.4 mM $MgCl_2$, 48 mM DTT, 100 μM ATP, in a total volume of 1 mL at 25 °C for 30 min. To remove the enzyme from phosphorylated protein B23 the sample was loaded to DEAE-Sephacel column and washed with 50 mM MES buffer (pH 6.2). The eluted CKII was monitored by autophosphorylation of CKII as detected by liquid scintillation counting. Protein B23 was eluted from the column with 50 mM Tris/HCl buffer containing 150 mM NaCl. For the cdc2 kinase phosphorylation the B23 samples were incubated at 25 °C for 45 min in a buffer containing 0.1 μg of cdc2 kinase, 50 mM Tris-HCl (pH 7.4), 0.4 mM $MgCl_2$, 1 mM EGTA, 200 μM ATP, and 1 μg/mL each of aprotinin, leupeptin, and pepstatin. After phosphorylation the samples were dialyzed in microdialysis flow cells against 20 mM HEPES buffer (pH 7.4). Aliquots (50 μL) from the incubation mixtures supplemented with γ-[^{32}P]ATP were routinely used to measure the efficiency of the enzyme reactions and to estimate the number of phosphoryl groups incorporated. Termination of phosphorylation in these aliquots was achieved by addition of 10% TCA followed by two more washes in 10% TCA, with subsequent washes with methanol and ether. The radioactivity incorporated into the protein was measured in a liquid scintillation counter. The number of phosphoryl groups incorporated into the protein was calculated from the radioisotope incorporation, the specific activity of the γ-[^{32}P]ATP, and the amount of protein as determined by the Bio-Rad protein assay. Approximately 1.5 or 1 phosphoryl groups per mole of protein were incorporated using CKII or cdc2 kinase, respectively.

Preparation of Labeled Import Substrates. The synthetic peptide containing the SV40 T antigen NLS was conjugated to BSA using the heterobifunctional cross-linking agent MBS (*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester) using a modification of a previously described method (Lanford et al., 1986; Kitagawa & Aikawa, 1976). The protein substrates (Rev and NLS−BSA) used for nuclear uptake studies were labeled with ^{125}I using Iodogen as described by the manu-

facturer (Pierce Chemical Co.). The labeled protein was separated from the free ^{125}I by a Sephadex G-25 column. The concentrations and specific activities of the ^{125}I-labeled proteins were determined by absorbance at 280 nm. Radioactivity was measured in a γ counter.

Isolation of Nuclei and Cytosol Fractions. Nuclei were prepared from rat liver, as described by Blobel and Potter (1966), with minor modifications. The homogenization buffer (TKM) contained 250 mM sucrose, 50 mM Tris, pH 7.6, 25 mM KCl, 2.5 mM $MgCl_2$, and 10 μg/mL each of leupeptin and pepstatin. Two different sources for cytosol were used for nuclear import assay: rabbit reticulocyte lysate (Promega Biotech, Milwaukee, WI) as previously described (Adam et al., 1991) and rat liver cytosol. Fresh rat liver was gently homogenized with 2 vol of import buffer (50 mM Tris, pH 7.6, 25 mM KCl, 2.5 mM $MgCl_2$, and containing 1 μg/mL each of leupeptin, pepstatin, and aprotinin) using a Potter homogenizer with a Teflon pestle. The homogenate was centrifuged at 1500*g* for 20 min. The supernatant was centrifuged at 15000*g* for 20 min, followed by 100000*g* for 1 h. The 100000*g* supernatant was then dialyzed for 4 h against import buffer.

Nuclear Import Assay. Nuclei were added to import buffer 50 mM Tris, pH 7.6, 25 mM KCl, 2.5 mM $MgCl_2$ containing 5 mM ATP, and 2 mg/mL of BSA to a final concentration of 10^7 nuclei/mL and allowed to equilibrate for 5 min at 30 °C as described by Dean and Kasamatsu (1994). After 5 min, ^{125}I-protein import substrate was added to the equilibrated nuclei. The typical import mixture was 260 μL. At various times after mixing, a 50 μL sample was removed and filtered through a 5.0 μm Durapore filter (diameter: 13 mm), which was presoaked for 4−5 h in import buffer containing 20 mg/mL of BSA to block protein-binding sites. The filter was washed with 10 mL of cold import buffer containing 2 mg/mL BSA, dried, and counted in a γ counter.

Microscopy. Nuclei were viewed under a Zeiss epifluorescence microscope equipped with a camera system.

Chemicals and NLS Peptide. The SV40 large T antigen NLS sequence (PKKKRKVEDPYC) was synthesized by Coast Scientific. MBS (*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester) was from Pierce Chemical Co. (Rockford, IL) product. All other chemicals were purchased from Sigma.

RESULTS

Nuclear Import of NLS−BSA and HIV-1 Rev. To determine the possible involvement of protein B23 in nuclear import we adapted the assay of Dean and Kasamatsu (1994) which measures uptake of labeled substrates into isolated nuclei by filtration onto nitrocellulose membranes. However, lower backgrounds were obtained if Durapore membranes were substituted for nitrocellulose. The former were used for the experiments described below. The import substrates were either NLS−BSA or the HIV-1 Rev protein which were labeled with ^{125}I. Nuclei were isolated from rat liver by centrifugation through a sucrose gradient and resuspended in TKM buffer which contained 0.24 M sucrose. Since the Dean and Kasamatsu (1994) assay was developed using nuclei of monkey kidney cells in culture, we did extensive evaluation of its use with rat liver nuclei. Microscopic examination indicated that less than 5% of the isolated nuclei were damaged and only traces of cytoplasmic debris were seen. The integrity of the nuclear membranes was judged by the criteria used by Dean and Kasamatsu (1994); exclusion of large fluorescently labeled macromolecules (FITC-dextran or FITC-albumin), inaccessibility by FITC-ConA to the lumen between the outer and inner nuclear membranes, and exclusive staining of the outer nuclear membrane by FITC-WGA. By these criteria more than 90% of the nuclei had intact nuclear envelopes.

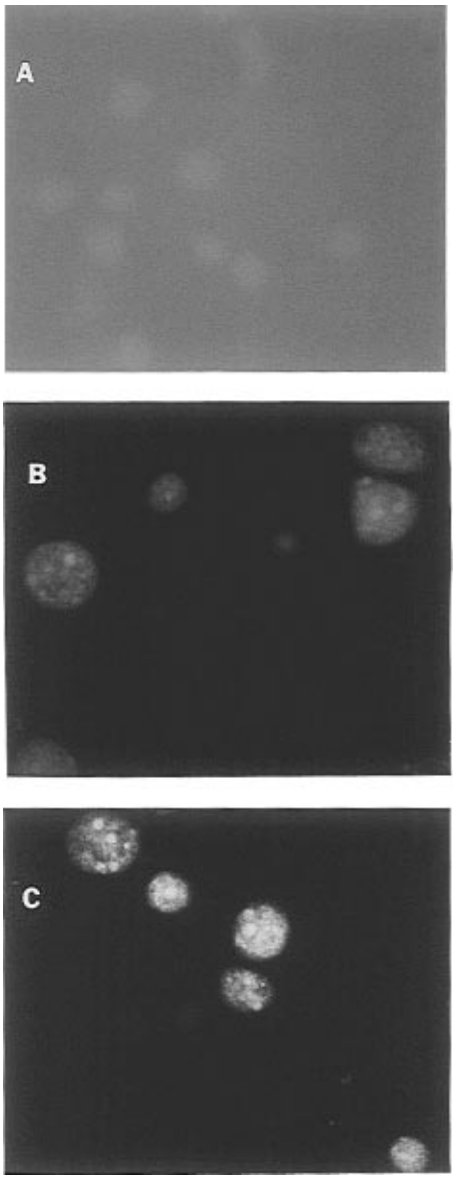

FIGURE 1: Import of fluorescently labeled Rev protein into isolated nuclei. Isolated rat liver nuclei were added (10^7 nuclei/mL) to TKM buffer containing 2 mg/mL of BSA and incubated at room temperature for 10 min with DTAF-labeled Rev protein (A) in the presence of 5 mM ATP (B) or 5 mM ATP and 0.1 μM protein B23.1 (C). The nuclei were then fixed (1 μL of 37% formaldehyde was added to a 10 μL sample), and the DTAF-labeled Rev protein was detected by fluorescence microscopy.

The nuclei were also examined after import of fluorescently labeled import substrate (Figure 1). Only traces of DTAF-labeled Rev were seen inside nuclei in the absence of ATP (Figure 1A). However, when 5 mM ATP was added to the system, the substrate was internalized in the nuclei and simply did not adhere to the surfaces (Figure 1B,C). The amount of internalized Rev appeared to be greater in the presence of protein B23.1 (Figure 1C).

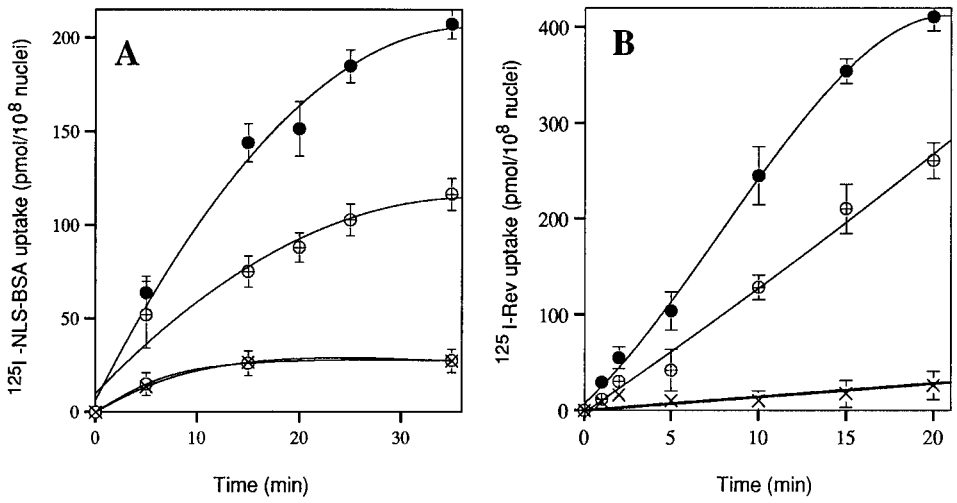

FIGURE 2: Import of ^{125}I-labeled substrates into isolated nuclei. Isolated rat liver nuclei were incubated with 25 nM [^{125}I]NLS−BSA (A) or 80 nM of [^{125}I]Rev protein (B) at 30 °C in TKM buffer (as described in Materials and Methods) in the presence (circled plus sign) or absence of 5 mM ATP (×). The assay was also performed with added protein B23.1 (1 μM) in the presence (●) or absence of ATP (○, panel A only). [^{125}I]BSA without NLS peptides attached was not measurably imported into the nuclei (data not shown). Each data point represents the mean ± SD of data obtained from five (A) or six (B) independent experiments.

To perform the assays the isolated nuclei were initially suspended in TKM buffer containing ATP and BSA and equilibrated for 5 min. The reaction was initiated by the addition of ^{125}I-labeled import substrate. After various times of incubation aliquots were removed, filtered, and counted for incorporated radioactivity. Figure 2A shows a typical time course of nuclear uptake of NLS−BSA. The uptake of NLS−BSA was ATP dependent, with essentially no uptake in the absence of ATP. The nuclei imported NLS−BSA with initial rates that were typically 5−6 pmol of NLS−BSA/min/10^8 nuclei at a ligand concentration of 25 nM. [^{125}I]BSA without NLS peptides attached was not measurably imported into the nuclei. The same nuclear import assay was used to measure the import of a second substrate, the HIV-1 Rev protein. Results similar to those obtained with NLS−BSA were seen with the Rev protein (Figure 2B). The import of radiolabeled Rev was also ATP dependent, with only low levels of incorporation if ATP was not present in the assay buffer. The addition of cytosolic fractions to the import buffer did not significantly alter the rate of import of either substrate (not shown).

Protein B23 Increases the Rates of Nuclear Import of NLS−BSA and the Rev Protein. The previously reported shuttling characteristics (Borer et al., 1989) and the affinity of protein B23 for peptides containing SV40 T-antigen NLS or Rev protein NOS sequences (Szebeni et al., 1995) raised the question of whether protein B23 may participate in the nuclear import of proteins. When recombinant protein B23.1 was added to the nuclear import system at a concentration of 6 μM the rate of import of NLS−BSA was increased approximately 2-fold (Figure 2A). Similarly, when Rev was used as the nuclear import substrate the presence of protein B23.1 (approximately 1:1 ratio of B23 to Rev) increased the rate of import of Rev approximately 2-fold (Figure 2B). Thus, protein B23 appears to have a similar stimulatory effect on the nuclear import of two different substrates.

Treatment of Isolated Nuclei with Anti-p97 or Wheat Germ Agglutinin Inhibits Nuclear Import. The first step in nuclear import is binding of the NLS to a receptor (p54/56) which binds to a 97 kDa protein (Adam & Adam, 1994). This complex of proteins then interacts with the nuclear pore complex via p97. Chi et al. (1995) have shown that an antibody to p97 inhibits nuclear import in permeabilized cells. To determine whether import in the isolated nuclear system was taking place by the same mechanism the nuclei were treated with a monoclonal antibody to p97. Isolated nuclei were preincubated for 5 min at 30 °C in the presence or absence of anti-p97 antibody in assay buffer containing ATP and then for 10 min with radiolabeled import substrates. Figure 3 shows that pretreatment with the antibody resulted in rates of import of either NLS−BSA or Rev similar to those seen with the control samples where ATP was omitted. In the presence of anti-p97, addition of recombinant protein B23.1 had no significant effect on the import of either substrate (Figure 3). When nuclei were treated with anti-p97 and observed by immunofluorescence microscopy, rings of fluorescence were observed around the nuclei (not shown). These results suggest that p97 is present in the isolated nuclear system and that both NLS−BSA and Rev are imported into nuclei by the same pathway described previously (Adam & Adam, 1994).

Wheat germ agglutinin (WGA) also inhibits import of proteins into the nucleus, probably by interaction with nucleoporins (Finlay et al., 1987). Experiments were performed to determine whether WGA affects import of the two substrates into isolated nuclei. Addition of increasing amounts of WGA to the import buffer caused a decrease in the uptake of NLS−BSA into the nuclei (Figure 4A) with greater than 90% inhibition at about 30 μg/mL. When this experiment was done in the presence of protein B23 a similar inhibition curve was seen (Figure 4B) indicating that protein B23 did not affect the interaction of WGA with the nuclei. Thus, this nuclear import system exhibits characteristics similar to those seen with systems utilizing permeabilized cells.

Stimulatory Effect of Protein B23 Is Saturable. Two important questions in determining the specificity of the stimulatory effect are whether the stimulation is saturable and if so, what is the stoichiometry with regard to the import substrate. These experiments were done with Rev as the import substrate because it contains only one NLS per molecule compared to the other substrate where multiple

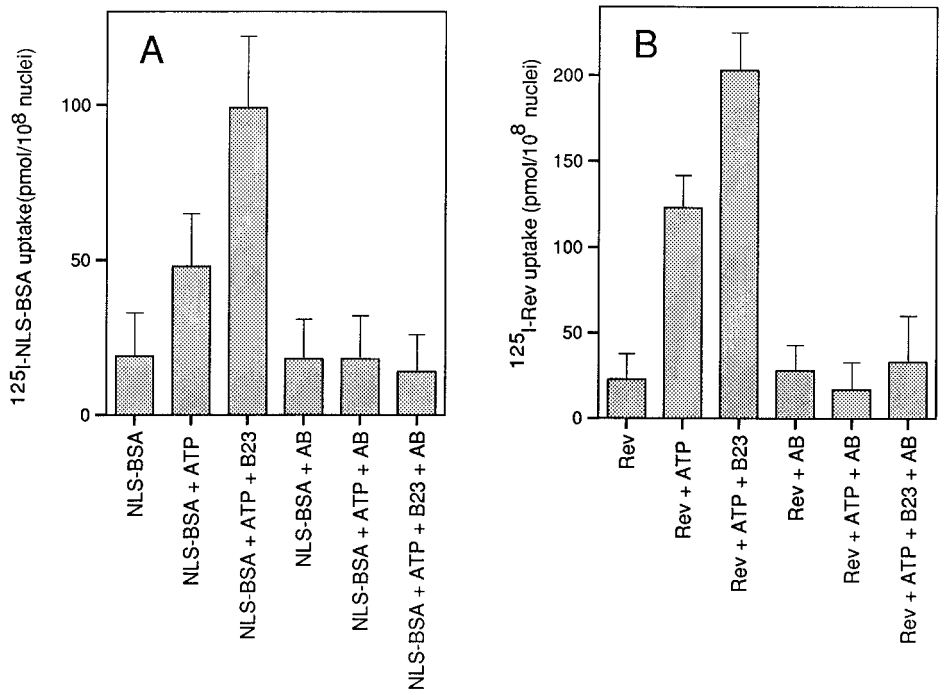

FIGURE 3: Effect of treatment of isolated nuclei with anti-p97 monoclonal antibody prior to nuclear import assay. Isolated rat liver nuclei were preincubated for 5 min at 30 °C in the presence or absence of 5 mM ATP and 50 μg/mL of anti-p97 antibody (AB) in TKM buffer and then for 10 min with 25 nM [^{125}I]NLS−BSA or 80 nM [^{125}I]Rev protein. Each value represents the mean ± SD from a minimum of six independent experiments.

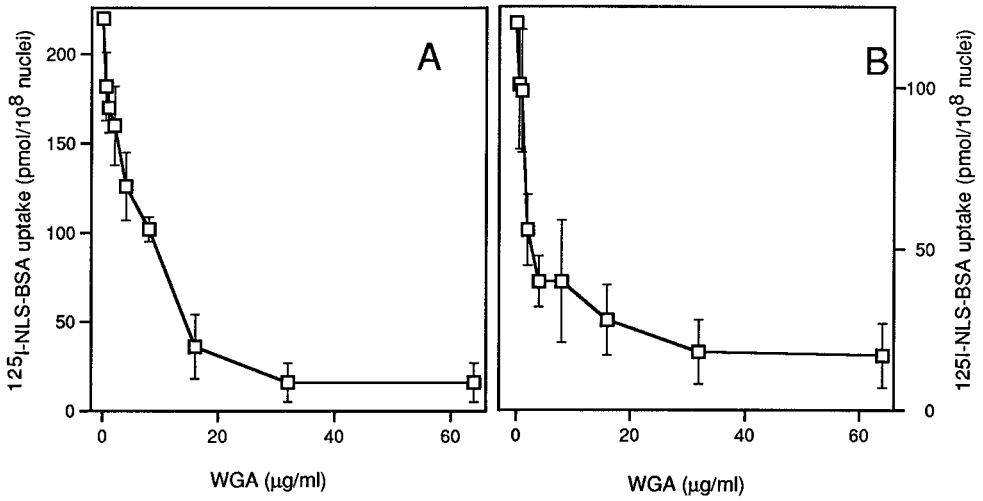

FIGURE 4: Effect of wheat germ agglutinin on uptake of import substrates into isolated nuclei. Isolated rat liver nuclei were preincubated for 5 min at 30 °C in the presence of 5 mM ATP and with the indicated concentration of WGA in TKM buffer and then for 10 min with 25 nM [^{125}I]NLS−BSA in the presence (panel A) or absence (panel B) of B23.1 (0.4 μM). Each data point represents the mean ± SD of data obtained from five independent experiments in panel A and three independent experiments in panel B.

NLS peptides are attached to BSA. Addition of increasing concentrations of protein B23.1 to the import system while maintaining constant concentrations of Rev causes increased rates of import up to a concentration of about 100−150 nM B23.1 (Figure 5). This is slightly above a 1:1 ratio of B23:Rev, suggesting that stoichiometric interaction of the two proteins provides optimal stimulation of import.

Phosphorylation of Protein B23.1 Further Enhances the Stimulation of Nuclear Import of NLS−BSA and Rev. We have previously shown that phosphorylation of protein B23 by casein kinase II enhances its affinity for the SV40 T- and Rev-derived NLS peptides (Szebeni et al., 1995). This effect was not seen with cdc2 kinase-phosphorylated B23. The major casein kinase II phosphorylation site in protein B23 (serine 125) is contained in the first highly acidic segment of the protein (Chan et al., 1990). Another potential site is located at threonine 185 in the second highly acidic segment. Since this region of the molecule is a proposed site of interaction with the NLS sequences we examined the effects of phosphorylation on the nuclear import of NLS−BSA and Rev. After phosphorylation of protein B23.1 by casein kinase II it was determined that 1.4−1.5 phosphoryl groups were incorporated per molecule of protein. This suggested complete phosphorylation of the major CKII site and partial phosphorylation of the second site. The presence of phosphorylated B23 increased the rate of nuclear import of either substrate 4−5-fold compared to assays done in the absence of added B23 (Figure 6). In the same series of experiments unphosphorylated B23.1 only produced a 2−3-fold enhancement over the controls.

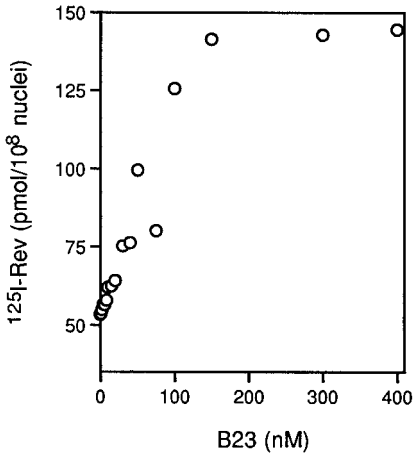

FIGURE 5: Effect of protein B23.1 concentration on Rev protein uptake into isolated nuclei. Isolated rat liver nuclei were preincubated for 5 min at 30 °C in TKM buffer containing 5 mM ATP as described in Materials and Methods. [^{125}I]Rev protein (100 nM) and various concentrations of protein B23.1 were added and incubated for 10 min, and the nuclei were collected on Durapore membranes as described in Materials and Methods.

Protein B23 is also known to be phosphorylated in the C-terminal region by a cdc2-type protein kinase (Peter et al., 1990). To determine whether the phosphorylation effect was due to phosphorylation in general or specific for a class of sites, protein B23.1 was also phosphorylated with a cdc2 protein kinase. Approximately one phosphoryl group per molecule of B23.1 was incorporated using the latter enzyme. When the cdc2 kinase-phosphorylated B23.1 was added to the assay buffer, the rate of import was stimulated 2−3-fold over control samples using either import substrate (Figure 6). In other words, there was no enhancement of nuclear import over that seen with unphosphorylated protein B23.1. This result suggests that the enhancement is only produced by phosphorylation at sites in the highly acidic segments of the center of the molecule but not by phosphorylation of cdc2-type sites in the C-terminal end.

Deletion or Mutation of the NLS of Protein B23.1 Abolishes Its Stimulatory Effect on Nuclear Import. Protein B23 contains a bipartite nuclear localization signal between the two acidic segments (Peculis & Gall, 1992). To test the possible role of the NLS in the stimulation of nuclear import by protein B23 a mutant form of the protein was prepared in which residues 141−157 were deleted (B23.1ΔNLS). When nuclear import experiments were done using this mutant protein there was no stimulation of import (Figure 7). In fact, the rate of import was slightly lower than when no B23 was added to the system. Because the deletion of 17 amino acid residues may cause a major change in the conformation of the protein a second NLS mutant protein was tested. In this case lysines 154, 155, and 157 were substituted with glutamines. This mutant protein (B23.1QNLS) was also incapable of stimulating import. This indicates that the NLS is essential for the stimulation of import and suggests that the effect depends on protein B23 itself being imported.

Proteins B23.1 and B23.2 Are Approximately Equal in Their Stimulatory Effects. Protein B23 is expressed in two isoforms, B23.1 and B23.2, differing only in their C-terminal ends. To determine whether the C-terminal end of the molecule is important for the stimulatory effect, the import assay was done in the presence or absence of equimolar amounts of either recombinant protein B23.1 or B23.2. Similar kinetics of import of Rev were obtained in the presence of either form of the protein (Figure 8). Furthermore, the additional stimulation of the rate of import obtained

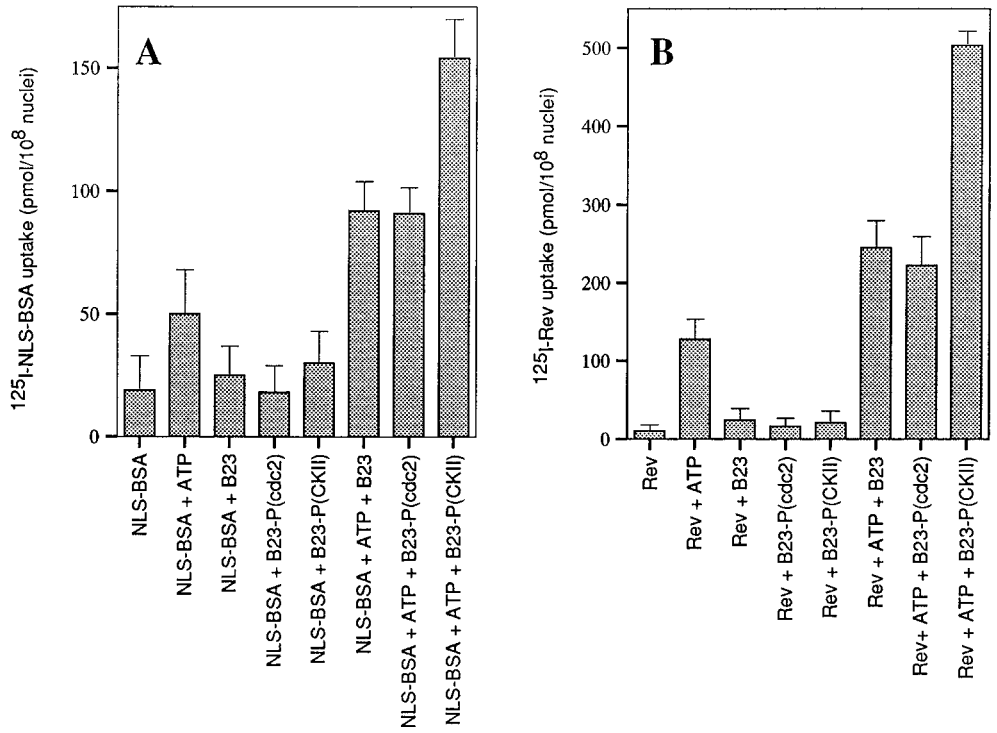

FIGURE 6: Effect of phosphorylation of protein B23.1 on uptake of NLS−BSA or the Rev protein into isolated nuclei. Isolated rat liver nuclei were incubated at 30 °C for 10 minutes in TKM buffer with 25 nM [^{125}I]NLS−BSA (A) with 80 nM of [^{125}I]Rev protein (B) or in the presence or absence of 5 mM ATP. The assay was also performed in the presence of 1 μM protein B23.1 or protein B23.1 phosphorylated with casein kinase II or the cdc2 kinase. After 10 min of incubation the mixtures were filtered through Durapore membranes as described in Materials and Methods. Each data point represents the mean ± SD of data obtained from three (A) or four (B) independent experiments.

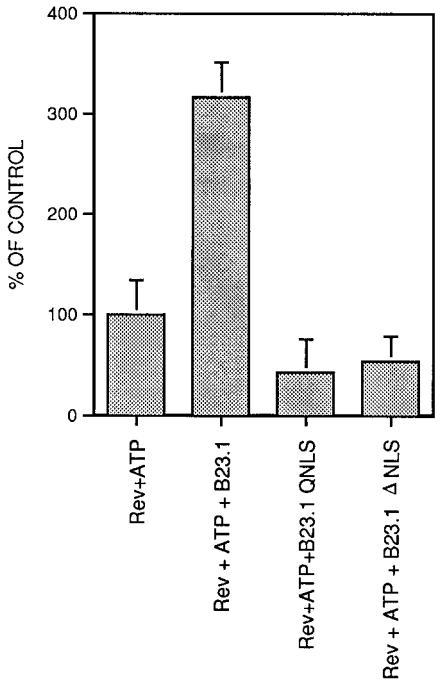

FIGURE 7: Effects of NLS mutations in protein B23.1 on nuclear uptake of the Rev protein. Isolated rat liver nuclei were incubated (as described in Figure 3) at 30 °C for 10 min in TKM buffer with 80 nM [^{125}I]Rev in the presence of 5 mM ATP. The assay was also performed in the presence of 0.4 μM protein B23.1 or the mutant proteins B23.1QNLS or B23.1ΔNLS. Each data point represents the mean ± SD of data obtained from three independent experiments. Data are expressed as the percentage of the control which was the labeled Rev uptake in the absence of added protein B23.1.

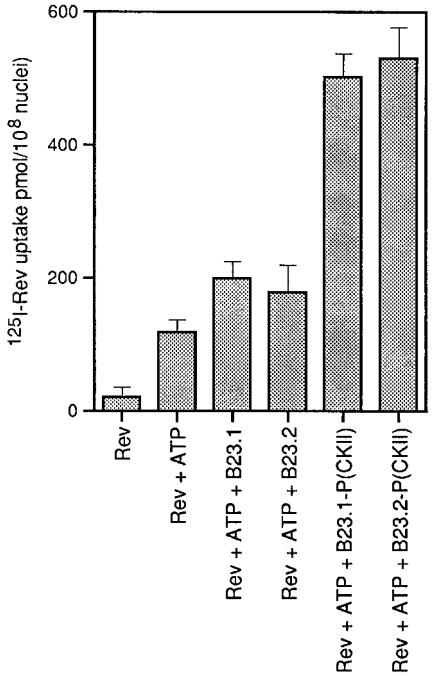

FIGURE 8: Effect of isoforms of protein B23 on uptake of the Rev protein into isolated nuclei. Isolated rat liver nuclei were incubated (as described in Figure 3) at 30 °C for 10 min in TKM buffer with 80 nM [^{125}I]Rev in presence or absence of 5 mM ATP. The assay was also performed in the presence of 0.4 μM protein B23.1 or B23.2. The values are means ± SD of data obtained from three independent experiments.

using CKII-phosphorylated proteins was essentially the same for both proteins B23.1 and B23.2. Thus, the carboxyl-terminal segment of the molecule must not be involved in the stimulation of import.

DISCUSSION

In the current work we have demonstrated that protein B23, especially in its phosphorylated form, stimulates the rate of nuclear import of two substrates: NLS−BSA and the HIV-1 Rev protein. The results were obtained using the nuclear import assay system developed by Dean and Kasamatsu (1994) employing isolated nuclei. This method was chosen because of the necessity for quantification of incremental changes in rates of uptake of substrates into nuclei. It is apparent from these studies that protein B23 is not absolutely required for import of either substrate, but rather it alters the rates of their import by the system that is present in nuclei. The assay employing filtration of isolated nuclei was preferred over fluorescence microscopy-based methods because of its relative simplicity and its utility for direct quantification of the uptake of radiolabeled substrates into nuclei.

The results presented here show that isolated rat liver nuclei imported the two NLS-containing substrates with all of the characteristics of nuclear import previously reported by Dean and Kasamatsu (1994). The incorporation of labeled substrate was ATP-dependent and inhibited by the lectin WGA. In addition, we showed that import was inhibited by treatment with an antibody to p97, an essential component of the nuclear import system (Adam & Adam, 1994). The latter experiment suggests that import is taking place in isolated nuclei via the system previously characterized using permeabilized cells. Furthermore, the substrates were shown to be internalized and did not simply adhere to the surfaces of nuclei. The nuclei used for the assay had intact envelopes and excluded large dextrans but allowed smaller dextrans to be taken up, indicating that import was not due to leakage of substrate into the nuclei. Thus, by several criteria, these nuclei imported NLS-containing substrates by the previously described pathway.

As indicated above, several characteristics of protein B23 have suggested that it plays a role in nuclear import; i.e., its affinity for peptides containing NLS sequences and its ability to shuttle between the nucleus and cytoplasm. Our more recent findings showed that there is specificity in the binding of NLS peptides to protein B23 and that this takes place at a 1:1 molar stoichiometry (Szebeni et al., 1995). Furthermore, the affinity of the NLS peptides for the protein is enhanced by casein kinase II phosphorylation. Taken together, these observations suggested the possibility that protein B23 affects the nuclear import of NLS-containing proteins. In the current work, we showed that protein B23 stimulates the rate of import of two different NLS-containing proteins, that maximal stimulation of import occurs when an import substrate (Rev) and B23 are at a 1:1 ratio and where B23 is phosphorylated by casein kinase II. These findings are all consistent with the previous observations, and they provide clues regarding the possible mechanism of this effect as discussed below.

Several scenarios can be proposed to explain the stimulation of the rate of nuclear import by protein B23: (a) direct stimulation of the import system without interacting with the NLS-containing protein, (b) providing an "anchor" for imported substrates in the nucleolus which prevents them

from being exported, (c) stimulation of the import system after interacting with the NLS-containing protein, (d) serving as a carrier of the imported protein which in some way accelerates the rate of import, and (e) altering the physical properties of the NLS-containing protein to enhance its import. The data we have presented suggest that direct interaction with the import substrate is required. In the case of the Rev protein where there is one NLS per protein molecule, maximal stimulation of import is seen at or above a 1:1 ratio of protein B23 to Rev in the import assay mixture. This is consistent with our previous findings where the NLS peptides bound B23 in a 1:1 stoichiometry. Furthermore, the rate of import is enhanced another 2-fold when B23 is phosphorylated with casein kinase II. Interestingly, this type of phosphorylation also increases the binding affinity of the NLS peptides approximately 2-fold. Thus, the stimulation of import correlates with the affinity of protein B23 for the NLS-containing protein and the possibility of stimulation without interaction as indicated in scenario a seems unlikely.

Experiments performed in this laboratory, but not shown here, suggest that the second scenario is also not likely to account for the stimulation. Nuclei were preincubated with assay buffer containing protein B23 and replaced with B23-free buffer prior to performing the assay. In these experiments there was no stimulation of import. Experiments with labeled B23 indicated that the exogenous B23 accumulated in nuclei and nucleoli. Thus, import of B23 into nuclei prior to import of another import substrate does not increase the rate of import. This reinforces the idea that protein B23 must interact with the import substrate outside of the nucleus prior to import into the nucleus.

None of the remaining three possibilities above is completely ruled out by the current data. However, there is some evidence to support both scenarios d and e. We showed that the NLS in B23 is required for stimulation of import, suggesting that B23 itself, or more likely, the B23−NLS protein complex is imported and for unknown reasons, accelerates the process. It is interesting that protein B23 has a bipartite NLS and the import substrates used to date have SV40 T-antigen type NLS sequences. It is tempting to speculate that the bipartite NLS is more efficiently imported and that import is stimulated by allowing the substrates to take advantage of the more efficient NLS by binding to a bipartite NLS-containing protein. However, we are not aware of any evidence suggesting enhanced import of proteins containing the bipartite NLS. With respect to scenario d, in unpublished studies we have shown that protein B23 is able to inhibit the temperature-dependent aggregation of the Rev protein reported by Wingfield et al. (1991). It is possible that the stimulatory effect is due to depolymerization of the Rev protein (or NLS−BSA) which should enhance the rate of import. In this case protein B23 could be acting as a molecular chaperone. Thus, scenario c or d or a combination of the two could account for the stimulation of import by protein B23.

The finding that the stimulation of nuclear import by CKII-phosphorylated protein B23 is greater than that seen with the unphosphorylated protein suggests that phosphorylation plays a role in the regulation of nuclear import. Protein B23 was shown to be a phosphoprotein more than two decades ago (Olson & Busch, 1974) and later shown to be a substrate for CKII (Pfaff & Anderer, 1987). It is interesting that phosphorylation at CKII sites in protein B23 is maximal during interphase but declines during mitosis when the CDC2 sites become phosphorylated (Peter et al., 1990). It is conceivable that CKII phosphorylation not only increases the affinity of B23 for other nucleolar components but also serves as a regulator of nuclear import which must be at a maximum rate during periods of high synthetic activity. This is consistent with recent studies by Vancurova et al. (1995) showing that CKII phosphorylation seems to be important for optimal nuclear import of nucleoplasmin, a protein closely related to B23. Recent work from this laboratory indicates that after mitosis the Rev protein does not enter the nucleus and accumulate in the nucleolus until G1 phase when active nuclear import is established and the nucleolus is fully operational (Dundr et al., 1996). The phosphorylation of protein B23 by CKII could be part of the regulatory process that causes the switch from a dispersed nucleolus and one that is actively accepting imported components and assembling them into preribosomal particles.

Although the precise functions of protein B23 are not clearly defined, the accumulated evidence suggest that it plays multiple roles in nucleolar function. In earlier studies it was shown to be a general nucleic acid binding protein (Dumbar et al., 1989). More recently, Herrera et al. (1995) showed that protein B23 is a ribonuclease, suggesting that it plays a role in preribosomal RNA processing. The current work suggests that it serves as an accessory factor in nuclear import, possibly of ribosomal proteins whose destination is the nucleolus. The HIV-1 Rev protein, which also localizes to the nucleolus (Cullen et al., 1993) may utilize this same pathway with the aid of protein B23. Future studies will be required to tie together these proposed functions and provide a unified picture for its participation in the nuclear and nucleolar machinery.

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