A Nuclear Export Signal Is Essential for the Cytosolic Localization of the Ran Binding Protein, RanBP1

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Abstract. RanBP1 is a Ran/TC4 binding protein that can promote the interaction between Ran and β-importin/β-karyopherin, a component of the docking complex for nuclear protein cargo. This interaction occurs through a Ran binding domain (RBD). Here we show that RanBP1 is primarily cytoplasmic, but the isolated RBD accumulates in the nucleus. A region COOH-terminal to the RBD is responsible for this cytoplasmic localization. This domain acts heterologously, localizing a nuclear cyclin B1 mutant to the cytoplasm. The domain contains a nuclear export signal that is necessary but not sufficient for the nuclear export of a functional RBD. In transiently transfected cells, epitope-tagged RanBP1 promotes dexamethasone-dependent nuclear accumulation of a glucocorticoid receptor–green fluorescent protein fusion, but the isolated RBD potently inhibits this accumulation. The cytosolic location of RanBP1 may therefore be important for nuclear protein import. RanBP1 may provide a key link between the nuclear import and export pathways.

T RAFFIC between the nucleus and the cytoplasm occurs through nuclear pore complexes in the nuclear membrane. Proteins containing a nuclear localization signal (NLS)[1] are actively transported into the nucleus by the nuclear import machinery. Several components involved in this process have been identified, and nuclear protein transport can be reconstituted in permeabilized cells that have been depleted of cytoplasm (for review see Melchior and Gerace, 1995). Nuclear protein export is not nearly as well understood, but the recent identification of nuclear export signals (NESs) may provide insight into the molecular basis for this process (for review see Gerace, 1995). NESs have been identified in three unrelated proteins thus far: protein kinase inhibitor (PKI) (Wen et al., 1995), HIV-1 Rev (Fischer et al., 1995), and heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) (Michael et al., 1995). The PKI and HIV-1 Rev NESs show limited similarity to one another, but the hnRNP A1 NES is distinctly different. All three proteins have been shown to shuttle between the nucleus and the cytoplasm and to mediate the export of other macromolecules. HIV-1

Rev and hnRNP A1 are involved in the export of RNA from the nucleus (Piñol-Roma and Dreyfuss, 1992; Fischer et al., 1994; for review see Izaurralde and Mattaj, 1995); PKI is a regulatory subunit of cAMP-dependent protein kinase A, entering the nucleus to escort nuclear cAMP-dependent protein kinase to the cytoplasm (Fantozzi et al., 1994).

The Ran GTPase is an abundant small guanine nucleotide binding protein found largely in the cell nucleus (Bischoff and Ponstingl, 1991a; Belhumeur et al., 1993) and is essential for nuclear protein import (Moore and Blobel, 1993; Melchior et al., 1993). Ran has also been implicated either directly or indirectly in other cell processes in various systems: cell cycle regulation (Ren et al., 1994; Kornbluth et al., 1994; Clarke et al., 1995), mRNA processing and export (Aebi et al., 1990; Forrester et al., 1992; Kadowaki et al., 1993; Schlenstedt et al., 1995a; Cheng et al., 1995), nuclear assembly and DNA replication (Dasso et al., 1992; Matsumoto and Beach, 1991), regulation of the yeast pheromone response (Clark and Sprague, 1989), chromosome stability (Matsumoto and Beach, 1991; Ouspenski et al., 1995), and nuclear protein export (Moroianu and Blobel, 1995).

RanBP1 and RanBP2 are putative downstream targets for Ran. They contain at least one Ran binding domain (RBD), which is responsible for the specific binding of Ran-GTP to these proteins (Beddow et al., 1995). RanBP2 is a 358-kD protein located at the nuclear pore and which contains four RBDs (Wu et al., 1995; Yokayama et al., 1995). Antibodies to RanBP2 are able to block nucleocytoplasmic transport (Yokayama et al., 1995), suggesting that the nucleoporin protein plays a role in this process.

1. *Abbreviations used in this paper:* CRS, cytoplasmic retention signal; GFP, green fluorescent protein; GR, glucocorticoid receptor; HA1, hemagglutinin; hnRNP A1, heterogeneous nuclear ribonucleoprotein A1; NES, nuclear export signal; NLS, nuclear localization signal; PKI, protein kinase A inhibitor; RanBP, Ran binding protein; RBD, Ran binding domain.

RanBP1 is an abundant 23.6-kD cellular protein that also binds Ran-GTP (Coutavas et al., 1993), but it is found primarily in the cell cytoplasm (Lounsbury et al., 1994; Schlenstedt et al., 1995b). The human RanBP1 behaves identically to the murine form in in vitro assays, and the two protein amino acid sequences are highly homologous (Bressan et al., 1991; Bischoff et al., 1995). The *Saccharomyces cerevisiae* RanBP1 homologue, Yrb1p, is 38% identical to the murine form, primarily in the region of the RBD. In *S. cerevisiae* RanBP1 is an essential gene, and may function in chromosome segregation during mitosis and maintenance of chromosome stability (Ouspenski et al., 1995) as well as nuclear protein import and mRNA export (Schlenstedt et al., 1995b). The in vivo association of the *S. cerevisiae* Ran and RanBP1 homologues, and the observation that the overexpression of either protein in *S. cerevisiae* results in the same phenotype further support the hypothesis that RanBP1 is an effector protein for Ran-GTP (Ouspenski et al., 1995).

RanBP1 stabilizes Ran-GTP in the presence of EDTA or the Ran nucleotide exchange factor, RCC1 (Lounsbury et al., 1994; Bischoff et al., 1995; Richards et al., 1995). RCC1 and RanBP1 do not appear to interact directly, but can form a ternary complex with nucleotide-free Ran (Bischoff et al., 1995; Hayashi et al., 1995). RanBP1 also possesses a unique regulatory activity; alone it does not stimulate GTP hydrolysis on Ran, but it does enhance the rate of GTP hydrolysis induced by the Ran GTPase activating protein (RanGAP) (Bischoff et al., 1995). The conserved RBD is also capable of binding to and stabilizing Ran-GTP, and of enhancing RanGAP-induced GTP hydrolysis in the absence of flanking regions (Beddow et al., 1995).

RanBP1 may have a role in nuclear protein import. Full-length RanBP1 and an isolated RBD are both capable of promoting the formation of a complex with Ran and β-importin/β-karyopherin, which is a component of the nuclear cargo docking complex (Lounsbury et al., 1996). RBDs may therefore act to link the docking and translocation steps of nuclear protein import. Temperature-sensitive *S. cerevisiae* RanBP1 mutants are defective in nuclear protein import at the nonpermissive temperature (Schlenstedt et al., 1995b), further implicating RanBP1 in this process. Thus far no functional difference has been seen for full-length RanBP1 and an isolated RBD. The role of the regions flanking the RBD in RanBP1 has not been addressed.

In this report we show that mammalian RanBP1 is cytoplasmic, but the isolated Ran binding domain from RanBP1 localizes to the cell nucleus. The nuclear localization of this domain is dependent on the ability to bind Ran-GTP. The sequence responsible for retaining RanBP1 in the cytoplasm is within a region COOH-terminal to the Ran binding domain (amino acids 161–189). This sequence contains an NES that is necessary but not sufficient to promote export of a functional RBD from the nucleus to the cytoplasm. The NES possesses limited similarity to that discovered in the protein kinase A inhibitor PKI, and mutations in hydrophobic residues of the RanBP1 NES affect protein localization. The isolated RBD blocks in vivo nuclear protein import in transiently transfected cells, suggesting that the cytoplasmic location of RanBP1 may be important to this process.

Materials and Methods

Construction of RanBP1 Fragments

Generation of RanBP1 fragments was accomplished using synthetic oligonucleotide primers in a PCR. The template DNA was wild-type RanBP1/HTF9A (a gift from J. Thorner) or RanBP1 that contained a point mutation in the Ran binding domain, E37K (Beddow et al., 1995). DNA products were subcloned into pKH³, a eukaryotic expression vector that encodes proteins with an NH_2-terminal triple hemagglutinin (HA1) tag (Mattingly et al., 1994). Plasmids for transfection were purified with columns (Qiagen Inc., Chatsworth, CA). Epitope-tagged human Δ151 cyclin B1 mutant was also generated using the PCR (a gift from J. Pines). To generate a Δ151 cyclin B1/RanBP1 COOH terminus or RBD/RanBP1 COOH terminus chimeras, a BglII site was created before the stop codon in Δ151 cyclin B1 or the RBD. A BamH1 site was introduced before the first codon of the COOH-terminal piece of RanBP1. The chimera was formed by the ligation of the BglII and BamH1 sites, resulting in a contiguous reading frame encoding Δ151 cyclin B1 or the RBD with amino acids from the COOH terminus of RanBP1.

For the production of recombinant GST-fusion proteins, DNA encoding the proteins was subcloned into the bacterial expression vector pGEX-2T (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). A fusion of GST with the S65T mutant of green fluorescent protein (GFP) (Heim et al., 1995) was generated by insertion of the GFP coding sequence into pGEX-2T. A GST-GFP-RanBP1 chimera was then generated by attaching selected RanBP1 sequences in frame at the COOH-terminal end of the GST-GFP coding sequence. Fusion protein expression was induced by the addition of 0.4 mM IPTG to DH5α cells transformed with the recombinant plasmid. Proteins were purified with glutathione S-Sepharose beads (Pharmacia LKB Biotechnology, Inc.) according to established methods, concentrated to 2–5 mg/ml, and stored in small aliquots at −80°C.

Transfection and Immunofluorescence

BHK21 (BHK clone 21) cells were maintained in DME supplemented with 10% calf serum (Hyclone, Logan, VT) at 37°C in a humidified atmosphere with 5% CO_2. Cells were plated onto two-well LabTek chamber dishes (Nunc, Roskilde, Denmark) and returned to the incubator for 24 h. Cells were transfected with 0.5 mg DNA per well for 18 h using $CaPO_4$ (Sambrook et al., 1989). Cells were rinsed and incubated in fresh media for 24 h. For immunofluorescent detection of the transfected protein, cells were fixed for 10 min with 4% paraformaldehyde in PBS and permeabilized on ice for 2 min with −20°C methanol. After blocking with 3% BSA in PBS for 30 min at 23°C, primary antibody (anti-HA1 ascites fluid 12CA5 [Boeringher Mannheim Biochemicals, Indianapolis, IN] at 1:200 dilution) in 3% BSA/PBS was added for 45 min at 23°C. After washing with 3% BSA/PBS, the secondary antibody (FITC-conjugated goat anti-mouse IgG at 1:500 [Jackson Immunoresearch Laboratories, West Grove, PA]) and 4,6-diamidino-2-phenylindole (DAPI) at 0.4 μg/ml were added in 3% BSA/PBS for 45 min at 23°C. After washing 5 times with 3% BSA/PBS, slides were mounted and sealed. Fluorescence was detected with an inverted microscope (Nikon Inc., Instrument groups, Melville, NY) at a magnification of 40. Confocal images were generated with a microscope (BX50; Olympus Corp., Precision Instruments Division, Lake Success, NY) at a magnification of 40 and a MRC-1000 laser scanning imaging system (Bio-Rad Laboratories, Richmond, CA).

Immunoprecipitation of HA1-tagged Proteins

BHK21 cells were transfected as described above, only using 10 μg DNA per 100-mm dish. Cells were rinsed twice with PBS and harvested in 0.5 ml lysis buffer (20 mM MOPS, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.5% Triton X-100, 0.1 mM PMSF, 25 μM aprotinin, and 25 μM leupeptin). Lysates were passed through a 26-g needle three times and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was added to 20 μl protein A–Sepharose beads (Sigma Chemical Co., St. Louis, MO) to which anti-HA1 antibody 12CA5 had been conjugated, and the mixture was mixed at 4°C for 1 h. Beads were then washed four times in lysis buffer. The immunoprecipitated proteins were separated on 12% SDS-PAGE gels and transferred to nitrocellulose.

Ran Overlays and Western Blots

Ran overlays were performed as described previously (Lounsbury et al.,

1994) with the following modification. Wild-type Ran in pGEX-2T was purified and thrombin cleaved as described previously (Richards et al., 1995). 24 pmol Ran was loaded with [α-^{32}P]GTP (3,000 Ci/ mM nucleotide; New England Nuclear, Boston, MA) and used to detect Ran-binding proteins in the overlay assay. Western blots were performed as previously described (Beddow et al., 1995). The nitrocellulose was blocked with 3% milk in TBS-T (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20). Primary monoclonal 12CA5 ascites fluid at 1:4,000 and the secondary HRP-conjugated goat anti–mouse IgG at 1:20,000 (Sigma Chemical Co.) were added in 3% BSA/TBS-T, and detection of specifically identified bands was by chemiluminescence.

Microinjection of GST Fusion Proteins

BHK21 cells were grown on CELLocate gridded coverslips (Eppendorf Inc., Fremont, CA). Cells were coinjected with purified GST fusion proteins and fluorescent dextran (Sigma Chemical Co.) in 10 mM sodium phosphate, pH 7.2, 70 mM KCl, and 1 mM MgCl$_2$, and were allowed to re-

cover for 1 or 2 h at 37°C in 5% CO$_2$. Cells were then fixed and processed for immunofluorescence with anti-GST antibodies and with FITC- or Cy^3-labeled secondary antibody (Jackson Immunoresearch Laboratories) as described above.

GR-GFP Plasmid Construction

The construction of pK7-GR-GFP, a glucocorticoid receptor–green fluorescent protein fusion expression vector, was constructed as described in Carey et al. (1996). Briefly, the triple HA1 epitope tag was excised from pKH^3, and the S65T GFP mutant (Heim et al., 1995) was introduced with cloning sites at the 5' and 3' ends of the GFP open reading frame. Glucocorticoid receptor (GR) was amplified by PCR and cloned into the XbaI site in pK7-GFP, 5' to the GFP open reading frame. This resulted in a contiguous coding sequence that was translated as a GR-GFP fusion driven by a cytomegalovirus promoter, and which obeys Kozak's rule for efficient translation initiation.
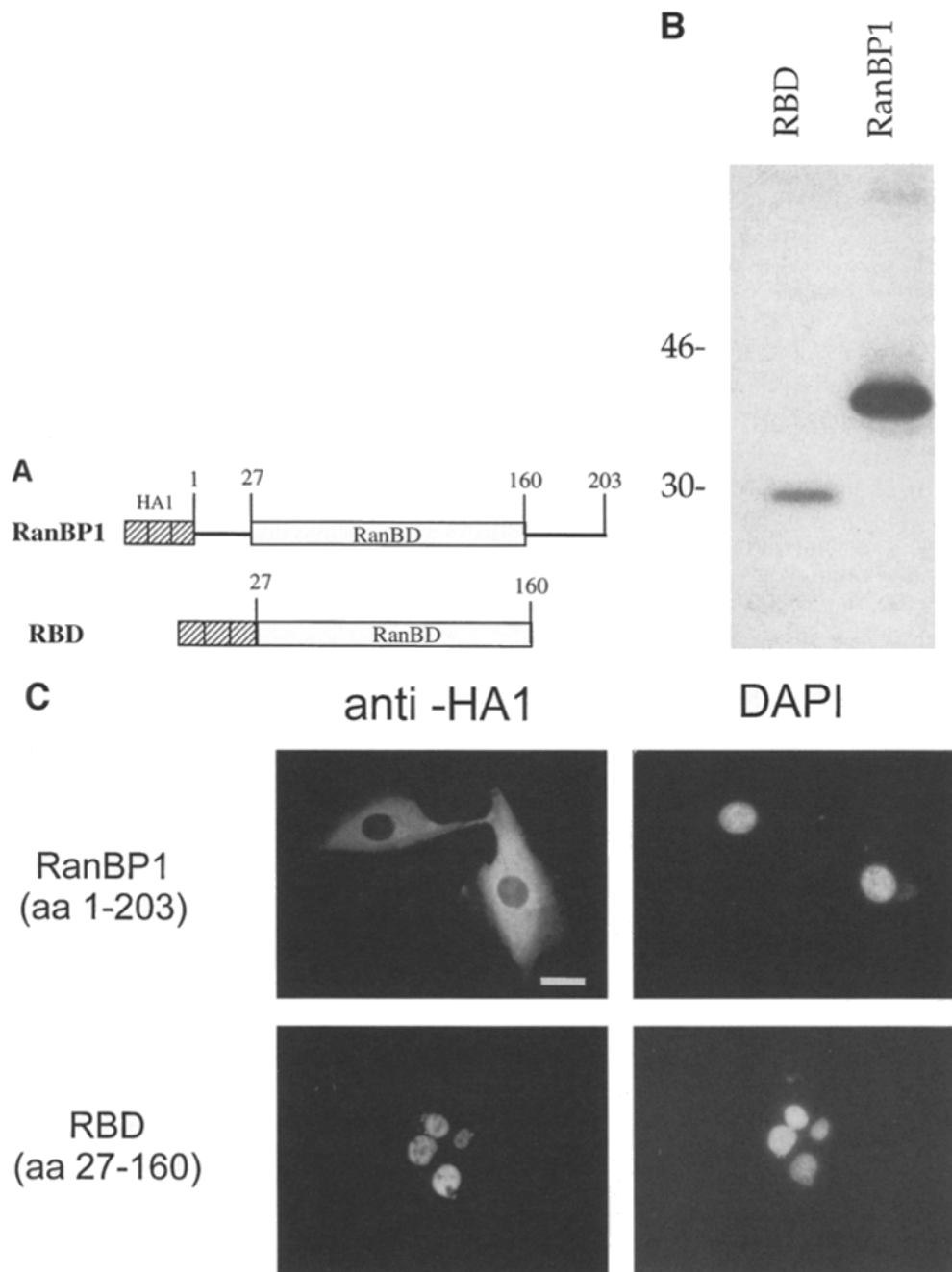

Figure 1. Subcellular localization of RanBP1 and the Ran binding domain. (A) Construction of epitope-tagged RanBP1 and isolated RBD. The hatched box represents a triple NH$_2$-terminal HA1 epitope tag. The numbering refers to amino acid residues in full-length RanBP1. (B) Ran-GTP overlay assay of epitope-tagged RanBP1 and RBD. BHK21 cells were transfected with pKH^3 RanBP1 or pKH^3-RBD, and immunoprecipitated with anti-HA1 antibody. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and overlaid with [α-^{32}P]GTP-Ran. (C) Immunofluorescent detection of epitope-tagged RanBP1 and isolated RBD. BHK21 cells, transfected as in B, were fixed and processed with anti-HA1 antibody. Epitope-tagged RanBP1 and RBD were visualized with FITC-conjugated secondary antibody, and nuclei were visualized by DAPI staining. Bar, 25 μm.
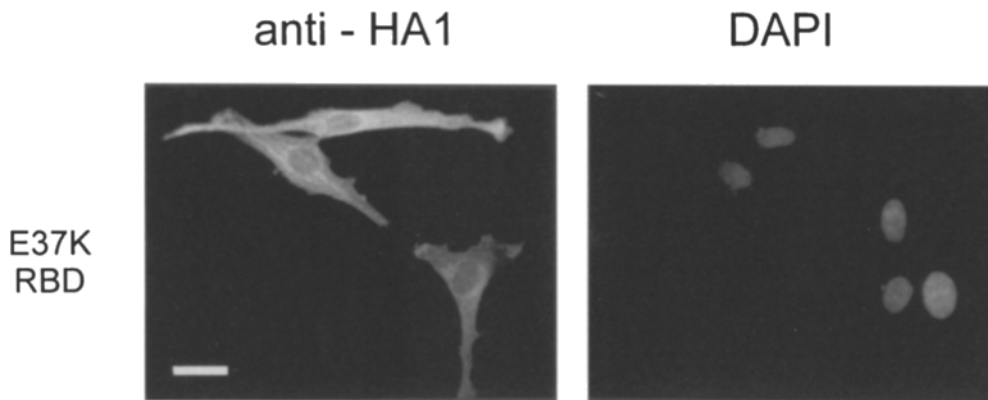
anti - HA1   DAPI

E37K
RBD

Figure 2. An RBD mutant that does not bind Ran is not localized to the nucleus. Immunofluorescent localization of E37K RBD. BHK21 cells were transfected with epitope-tagged RBD that contains a point mutation that decreases binding to Ran-GTP (Beddow et al., 1995). HA1-E37K RBD was visualized as described in Materials and Methods and in Fig. 1 C. Bar, 25 μm.

In Vivo Nuclear Import of GR-GFP

BHK21 cells were cotransfected with pK7-GR-GFP and various concentrations of pKH³-RanBP1 or pKH³-RBD, as described above and grown in DME with 10% glucocorticoid-stripped serum (Eckert and Katzenellenbogen, 1982). To reduce background fluorescence before microscopy, cells were incubated in 20 mM Hepes, pH 7.4, 125 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.2 mM KH_2PO_4, 5.6 mM glucose, and 1 mg/ml BSA. The agonist dexamethasone was added to 1 μM for the indicated times, whereupon cells were fixed with 4% paraformaldehyde in PBS. A Bio-Rad MC1000 confocal microscopy system was used to quantitate the nuclear/cytoplasmic ratio of GR-GFP. The number of pixels in the nuclei and cytoplasm of at least 10 cells were determined for each time point using software (Confocal Assistant; Bio-Rad Laboratories). No image enhancement was performed before quantitative analysis.

Results

Localization of the Full-Length RanBP1 and the Isolated Ran Binding Domain

The RBD of RanBP1 is flanked by 26 amino acids at the NH_2 terminus and 43 amino acids at the COOH terminus. The biochemistry of the isolated RBD is similar to that of RanBP1, but the function of the flanking regions is unknown. Therefore, we asked whether these flanking sequences affect the localization of RanBP1. HA1 epitope–tagged full-length RanBP1 and the isolated RBD of this protein were constructed and expressed in BHK21 cells (Fig. 1 *A*). The full-length protein is 203 amino acids in length, and the isolated RBD is comprised of amino acids 27–160. Immunoprecipitation of the epitope-tagged proteins from transiently transfected BHK21 cells and subsequent Ran-GTP overlay assay showed that the HA1-RanBP1 and HA1-RBD were expressed in these cells, and that the epitope tag did not interfere with the ability of these proteins to bind Ran-GTP (Fig. 1 *B*). (Note that the mobility of HA1-RanBP1 on SDS-PAGE gels is lower than predicted from its amino acid sequence [Coutavas et al., 1993]). By indirect immunofluorescence, HA1-RanBP1 was found to be cytoplasmic, and was excluded from the nucleus (Fig. 1 *C*). This observation is consistent with the location of the endogenous protein as determined by subcellular fractionation (Lounsbury et al., 1994), and with that of the yeast RanBP1 homologue, Yrb1p (Schlenstedt et al., 1995*b*). However, the isolated HA1-RBD of RanBP1 protein was exclusively nuclear (Fig. 1 *C*), suggesting that sequences outside the RBD are necessary for maintaining RanBP1 in the cytoplasm.

Evidence that Nuclear Accumulation of the RBD Requires Ran-GTP Binding

Both RanBP1 and the isolated RBD bind Ran-GTP. We therefore wished to determine if the nuclear accumulation of the RBD is dependent on Ran-GTP binding. To test this hypothesis, we used the RBD of a mutant RanBP1, that has a lysine substituted for glutamine at amino acid 37 (E37K). This protein has a greatly reduced affinity for Ran-GTP (Beddow et al., 1995). When the E37K RBD was localized by immunofluorescence, it was found diffusely throughout the cell (Fig. 2). Therefore, the localization of the RBD in the nucleus may depend on the ability of that domain to bind to Ran-GTP.

The Region COOH-terminal to the RBD Is Responsible for Cytoplasmic Retention of RanBP1

Because the isolated RBD of RanBP1 accumulated in the nucleus, the cytoplasmic location of the full-length RanBP1 must be controlled by either or both regions flanking the RBD. To discern which regions are necessary for cytoplasmic retention of RanBP1, we constructed various fragments of this protein, two of which have a truncated RBD (Fig. 3 *A*). Immunoprecipitation of the HA1 epitope–tagged constructs from transfected cells, with subsequent Ran-GTP overlay (Fig. 3 *B*) and Western blot (Fig. 3 *C*) showed that these proteins were expressed in the transfected cells, but as expected (Beddow et al., 1995) only those fragments with a full-length RBD were able to bind Ran-GTP.

Immunofluorescent detection of these HA1-RanBP1 fragments in transfected cells revealed that the NH_2-terminal 26 amino acids do not affect the nuclear accumulation of the RBD (Fig. 4 *A*) and, as expected from the result with the E37K RBD, nuclear accumulation was dependent on an intact RBD (Fig. 4, *B* and *D*). The COOH-terminal 43 amino acids of RanBP1, however, were capable of retaining a functional RBD in the cell cytoplasm (Fig. 4 *C*). Therefore, the COOH-terminal 43 amino acids of RanBP1 are necessary and sufficient for the cytoplasmic localization of RanBP1.

COOH Terminus of RanBP1 Can Function in a Heterologous Context

One mechanism by which the COOH terminus may act to exclude RanBP1 from the nucleus is through nonspecific
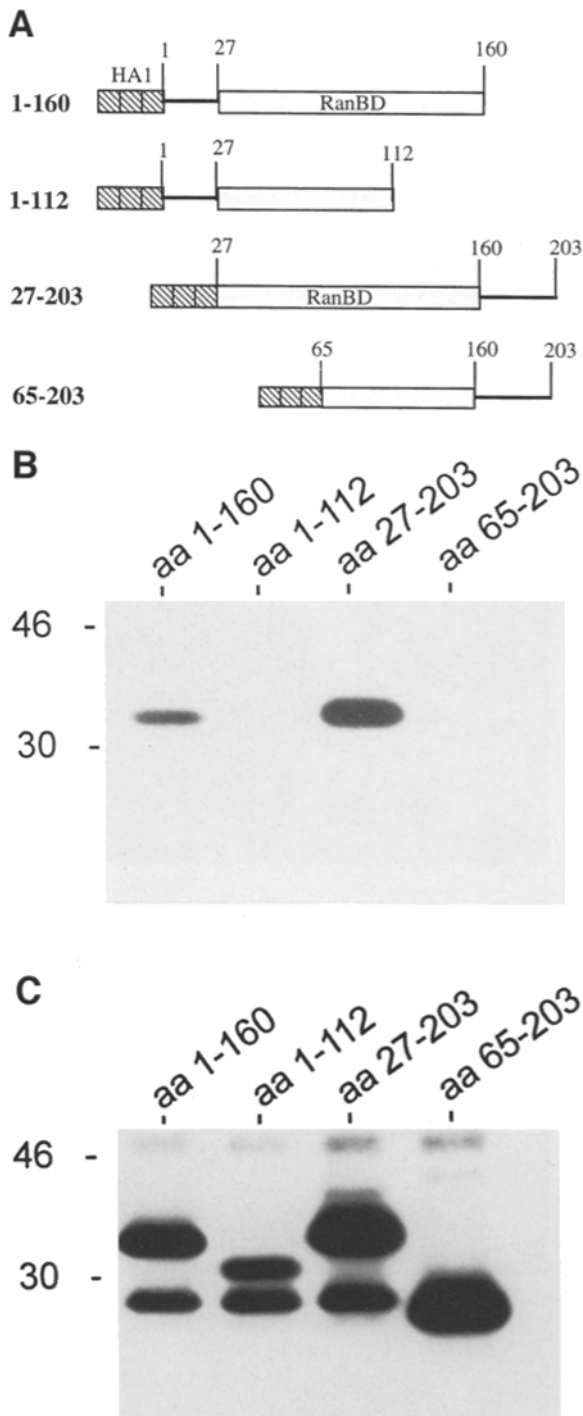
A

B

C

Figure 3. Construction and characterization of RanBP1 fragments. (*A*) Schematic representation of RanBP1 fragments. Fragments were constructed and subcloned into pKH³. Hatched boxes represent the triple HA1 epitope tag. Numbers refer to amino acid residues of full-length RanBP1 (Coutavas et al., 1993). (*B*) Ran-GTP overlay of epitope-tagged RanBP1 fragments. BHK21 cells were transfected with 10 μg DNA, lysed, and immunoprecipitated with anti-HA1 antibody. Immunoprecipitates were separated by 12% SDS-PAGE, transferred to nitrocellulose, and overlaid with [α-³²P]GTP-Ran. (*C*) Western blot of epitope-tagged RanBP1 fragments. Immunoprecipitated epitope-tagged RanBP1 fragments from transfected BHK21 cells were separated, transferred to nitrocellulose, and blotted with anti-HA1 antibody to show that the fragments are expressed in cells. Note the 25-kD immunoglobulin light chain is present in all lanes.

steric hindrance. The COOH terminus could sterically impede the interaction of the RBD with a nuclear component and block nuclear accumulation. Alternatively, the COOH terminus could contain a sequence-specific cytoplasmic retention signal (CRS) or NES that maintains the protein in the cytoplasm. To investigate these possibilities, we first constructed a RBD that had the 33–amino acid epitope tag on the COOH terminus instead of the NH₂ terminus. As shown in Fig. 5, this RBD still localized to the cell nucleus, demonstrating that the presence of an unrelated amino acid sequence COOH-terminal to the RBD is not sufficient to exclude the RBD from the nucleus.

A cytoplasmic retention or nuclear export signal would be expected to function independently of the protein context in which it is inserted. To test for the ability of the RanBP1 COOH terminus to act in a heterologous context, we used a mutant form of human cyclin B1. Cyclin B1 is normally found in the cell cytoplasm, a consequence of a possible CRS within residues 81–151 (Pines and Hunter, 1994). A mutant cyclin B1, lacking the first 151 amino acids, accumulates in the nucleus despite the lack of a nuclear localization signal (Fig. 6, Pines and Hunter, 1994). However, when amino acids 161–203 of RanBP1 were attached to the COOH terminus of this mutant cyclin B1, the chimeric protein was cytoplasmic (Fig. 6). From these data, we conclude that the COOH terminus of RanBP1 is capable of localizing non–NLS-containing nuclear proteins to the cytoplasm. Furthermore, this function is not a result of specific interactions with the RBD, and suggests that the COOH terminus contains a signal sequence such as a NES or a CRS.

Cytoplasmic Localization of RanBP1 Is Mediated by Amino Acids 161–189

To further define the region of RanBP1 necessary for cytoplasmic retention, we made a series of deletion mutants and point mutations in HA1-RanBP1 and localized the mutant proteins in transiently transfected BHK21 cells. Point mutations were introduced into the COOH terminal region of RanBP1 to identify individual residues important for the retention of RanBP1 in the cytoplasm (Fig. 7). Changes in acidic residues D174, E184, and E194 had no effect; HA1-RanBP1 remained in the cytoplasm. However, changes in hydrophobic residues L186 and V188 resulted in the nuclear localization of the mutant HA1-RanBP1 proteins.

Cytoplasmic localization of HA1-RanBP1 was unaffected by the removal of amino acids 190–203 (Fig. 7). Deletion of the COOH terminus from amino acid residues 180 and 186 resulted in a mixed population, in which some cells expressed nuclear HA1-RanBP1 while others displayed HA1-RanBP1 predominantly in the cytoplasm. The removal of amino acids 178–203 from RanBP1 led to nuclear accumulation of the deletion mutant in all transfected cells (Fig. 7). From these data, we conclude that amino acids 178–189 of RanBP1 are essential for the cytoplasmic localization of RanBP1.

To ascertain whether this region of RanBP1 is sufficient for relocalization of the RBD from the nucleus to the cytoplasm, amino acids 178–189 from RanBP1 were attached directly to the HA1-RBD. This fusion protein localized to
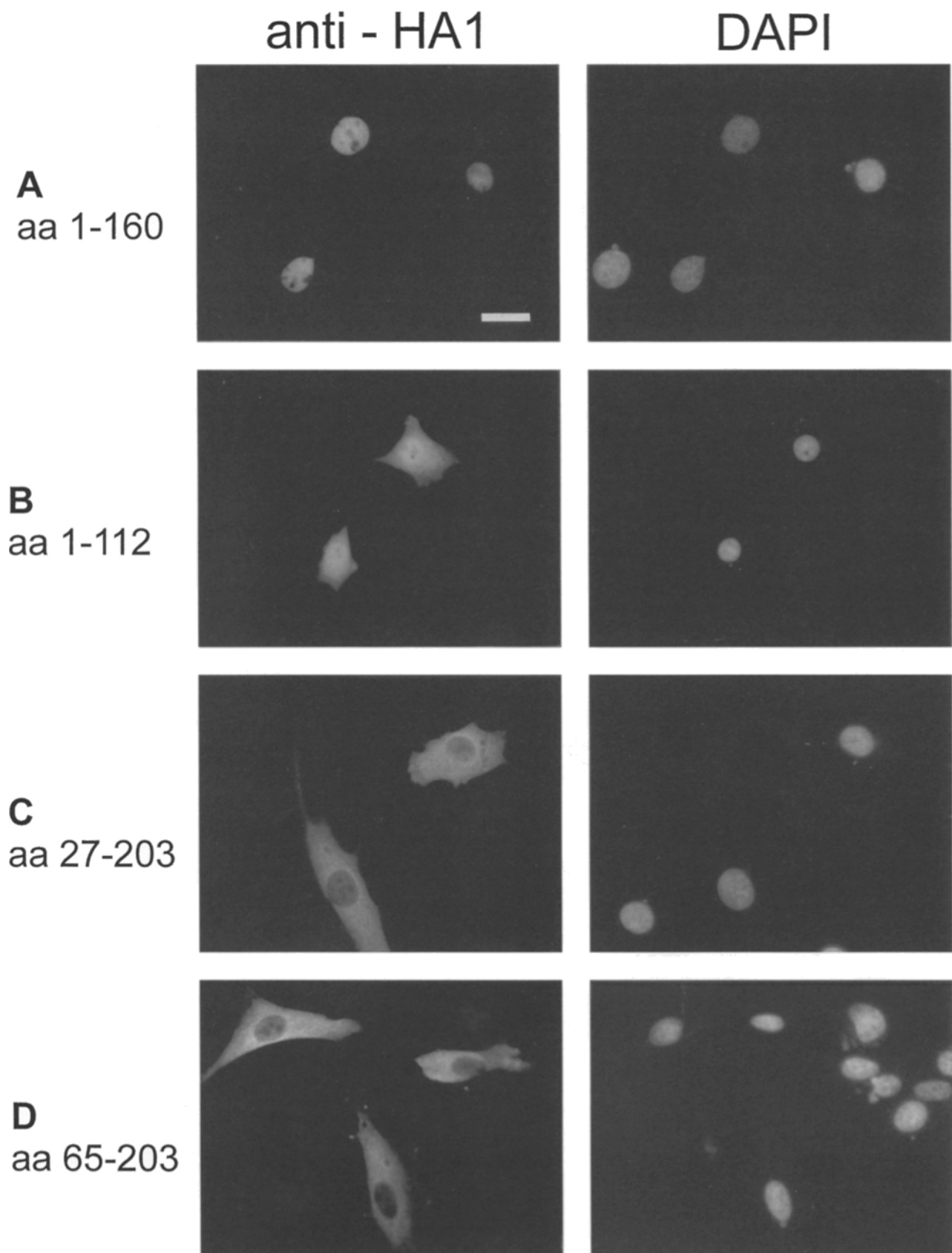
Figure 4. Immunofluorescent localization of epitope-tagged RanBP1 fragments. HA1-tagged RanBP1 fragments from Fig. 4 were transfected into BHK21 cells and detected by indirect immunofluorescence with anti-HA1 antibody, with nuclei localized by DAPI staining. Fragments used were (*A*) aa 1–160, (*B*) aa 1–112, (*C*) aa 27–203, (*D*) aa 65–203. Bar, 25 μm.
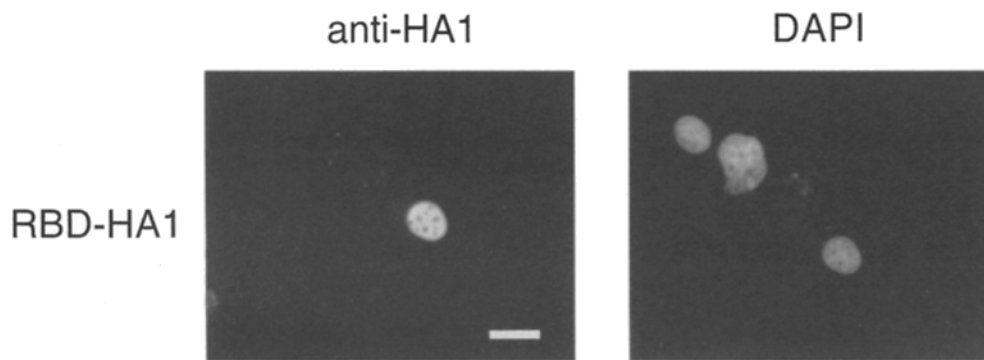
anti-HA1

DAPI

RBD-HA1

Figure 5. Localization of COOH-terminal epitope-tagged RBD. The RBD was amplified using synthetic oligonucleotide primers and subcloned into pKH[3] so that the triple HA1 tag was on the COOH terminus of the RBD rather than the NH_2 terminus. BHK21 cells were transfected and processed for immunofluorescence with anti-HA1 antibody and DAPI as described in Materials and Methods. Bar, 25 μm.

the nucleus (Fig. 8). To determine if the binding of the RBD to nuclear Ran-GTP overrides the ability of amino acids 178–189 to cytoplasmically localize the RBD, amino acids 178–189 were attached to the E37K RBD. By indirect immunofluorescence, E37K RBD with amino acids 178–189 at its COOH terminus was cytoplasmic (data not shown), suggesting that the binding of the RBD to nuclear Ran-GTP dominantly interferes with the ability of RanBP1 amino acids 178–189 to localize this protein to the cytoplasm. Therefore, amino acids 178–189 of RanBP1 are necessary but not sufficient to retain a functional RBD in the cell cytoplasm.

RanBP1 Contains a Nuclear Export Signal

Cytoplasmic localization of RanBP1 could be attained either by cytoplasmic retention or by nuclear export; either mechanism requires a specific signal sequence. We therefore wished to determine whether RanBP1 is exported from the nucleus, and whether the region between amino acid residues 178–189 functions independently as an NES.

GST fusion proteins were injected into BHK21 cell nuclei or cytoplasm. Fluoroscent dextran was coinjected with the protein and, because it is too large to diffuse across the nuclear membrane, was used to mark the original injection

site. Cells were incubated at 37°C, 5% CO_2, then processed for immunofluorescence with anti-GST antibody to localize the GST fusion protein. As shown in Fig. 9, the full-length GST-RanBP1 translocated from the nucleus to the cell cytoplasm within 1 h after injection into the nucleus. The COOH-terminally deleted GST-RanBP1 (1–160), however, remained in the nucleus. When injected into the cytoplasm, GST-RanBP1 remained cytoplasmic (Fig. 9), but GST-RanBP1 lacking the COOH terminus (aa 1–160) was diffuse and was not excluded from the nucleus. When GST fusions of RanBP1 point mutants L186A and V188A were injected into the nucleus they remained nuclear (data not shown). These results suggest that the COOH terminus of RanBP1 contains an NES.

To determine whether this NES can function independently in a heterologous context, microinjections were performed using GST-GFP fusions. At 52 kD, GST-GFP is above the passive diffusion limit for passage through the nuclear pore (Davis, 1995). GFP alone is diffusely expressed in transiently transfected BHK21 cells (Carey et al., 1996); therefore, GFP does not appear to contain any signal sequences such as a NLS or NES that would interfere with the assay. When injected into the nucleus, GST-GFP remained nuclear (Fig. 10). However, when RanBP1 amino acids 178–189 were expressed at the COOH termi-
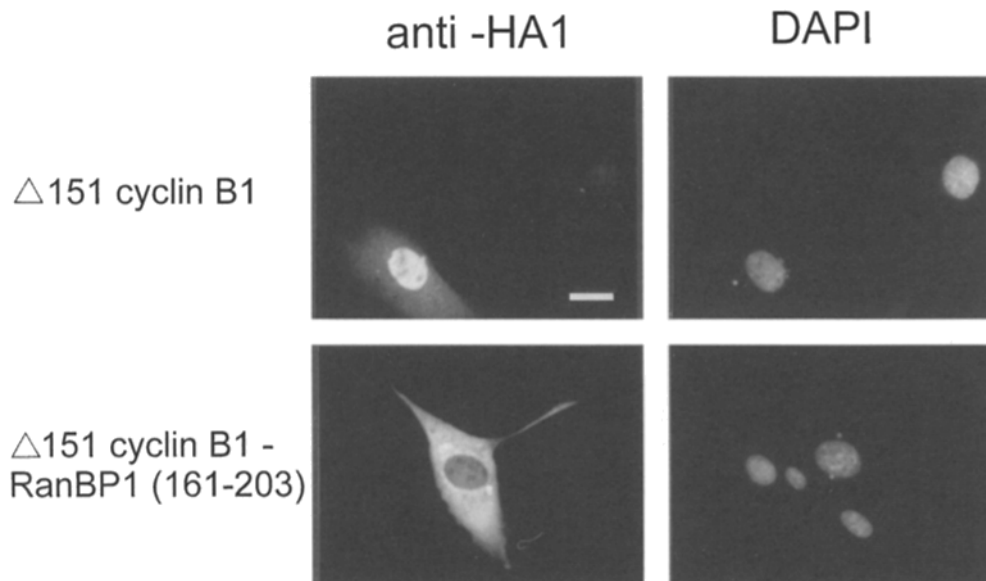
anti -HA1

DAPI

△151 cyclin B1

△151 cyclin B1 - RanBP1 (161-203)

Figure 6. Localization of △151 cyclin B1-RanBP1 chimera. The COOH-terminal region of RanBP1 (aa 161–203) was ligated to the COOH terminus of △151 cyclin B1 using complementing BglII and BamH1 sites. This construct and △151 cyclin B1 were subcloned into pKH[3]. BHK21 cells were transfected and processed for immunofluorescent detection of the epitope tag as described in Materials and Methods. Bar, 25 μm.

POINT MUTANTS

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                ↓174        ↓184        ↓194
aa 161-203  EIEEREKKGPGKNDNAEKVAEKLEALSVREAREEAEEKSEEKQ
                        186 ↑ ↑188
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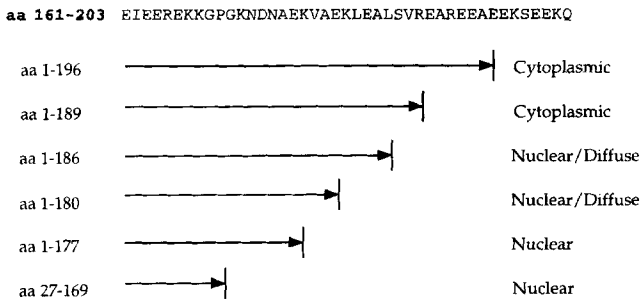| Mutation	Location
D174K	Cytoplasmic
E184K	Cytoplasmic
L186A	Nuclear
V188A	Nuclear
E194K	Cytoplasmic

DELETION MUTANTS

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aa 161-203  EIEEREKKGPGKNDNAEKVAEKLEALSVREAREEAEEKSEEKQ
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aa 1-196	Cytoplasmic
aa 1-189	Cytoplasmic
aa 1-186	Nuclear/Diffuse
aa 1-180	Nuclear/Diffuse
aa 1-177	Nuclear
aa 27-169	Nuclear

Figure 7. Mutation analysis of RanBP1 COOH terminus. Mutations were generated by PCR mutagenesis and the products were cloned into pKH³. Localization of HA1-tagged proteins in transiently transfected BHK21 cells was determined by indirect immunofluorescence as described in Materials and Methods.

nus of GST-GFP, protein injected into the nucleus was translocated to the cytoplasm.

These data confirm the presence of a nuclear export signal in the COOH terminus of RanBP1, requiring the sequence ^{178}KVAEKLEALSVR^{189}. However, fusion of this sequence to a functional RBD (RBD + aa 178–189) was not sufficient to export this protein from the nucleus (Fig. 9). When injected into the cytoplasm, the (RBD + aa 178–189) fusion was not exclusively cytoplasmic (Fig. 9). These results are in agreement with those obtained by transient transfection of the HA1-tagged version of this protein

(Fig. 8). Overall, the results demonstrate that residues 178–189 of RanBP1 function as an NES, and that this sequence is necessary but not sufficient for the cytoplasmic localization of RanBP1.

Nuclear import of GR-GFP Is Inhibited by Expression of an Isolated RBD

In vitro, RanBP1 can promote the interaction of Ran with β-importin/β-karyopherin, a cytosolic component of the nuclear protein import docking complex (Lounsbury et al., 1996). The isolated RBD of RanBP1 or RanBP2 also promotes this interaction. Therefore, it was of interest to determine the effect of a RBD on nuclear protein import. To examine the nuclear transport of physiological cargo in intact cells, we used a fusion protein of the GR with GFP (Fig. 11 *A*). Expression of the GR-GFP fusion is readily detectable in the cytoplasm of individual living cells within 24 h after transfection. After addition of the agonist dexamethaxone, the fusion protein accumulates in the nucleus with kinetics similar to those described for the glucocorticoid receptor alone (Carey et al., 1996).

To examine the effects of RanBP1 and the isolated RBD on the dexamethasone-induced nuclear accumulation of GR-GFP, BHK21 cells were cotransfected with pK7-GR-GFP and pKH³-Ran BP1 or pKH³-RBD. Expression of HA1-RanBP1 did not inhibit nuclear transport, but instead produced a small, concentration-dependent increase in the nuclear/cytoplasmic ratio of GR-GFP (Fig. 11, *B* and *C*). This result is consistent with a role for RanBP1 in the nuclear transport function of the Ran GTPase. However, the isolated HA1-RBD dominantly inhibited the nuclear accumulation of GR-GFP at all concentrations tested (Fig. 11, *B* and *C*), suggesting that the cytoplasmic localization of RanBP1 may be important for nuclear protein transport.

Discussion

The Ran binding protein RanBP1 is an abundant, cytoplasmic protein that can stabilize the Ran GTPase in the GTP-bound state, enhance RanGAP activity (Bischoff et
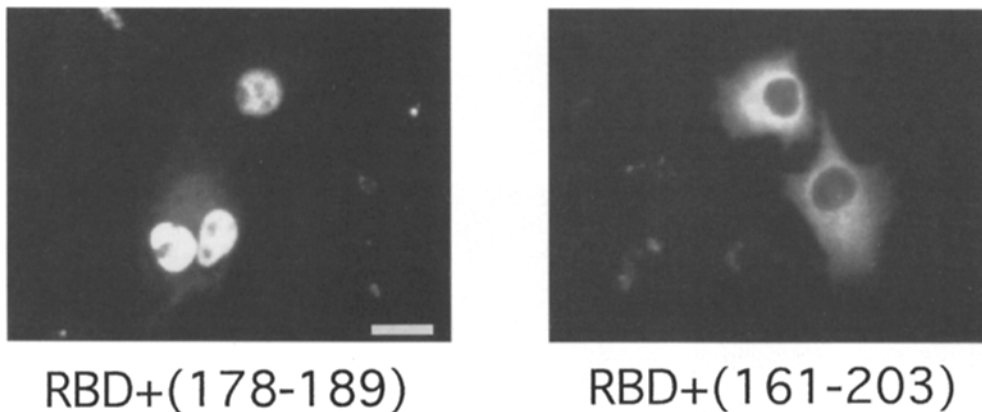

RBD+(178-189)

RBD+(161-203)

Figure 8. Subcellular localization of RBD + (178–189) RanBP1 fusion. Amino acids 178–189 or 161–203 of RanBP1 were attached to the isolated RBD as was described for the Δ151 cyclin B1 chimera (Fig. 7, Materials and Methods). The constructs were subcloned into pKH³ and transfected into BHK21 cells. HA1-tagged proteins were detected by indirect immunofluorescence as described previously. Shown are confocal images of HA1-tagged proteins in these transfected cells. Bar, 25 μm.
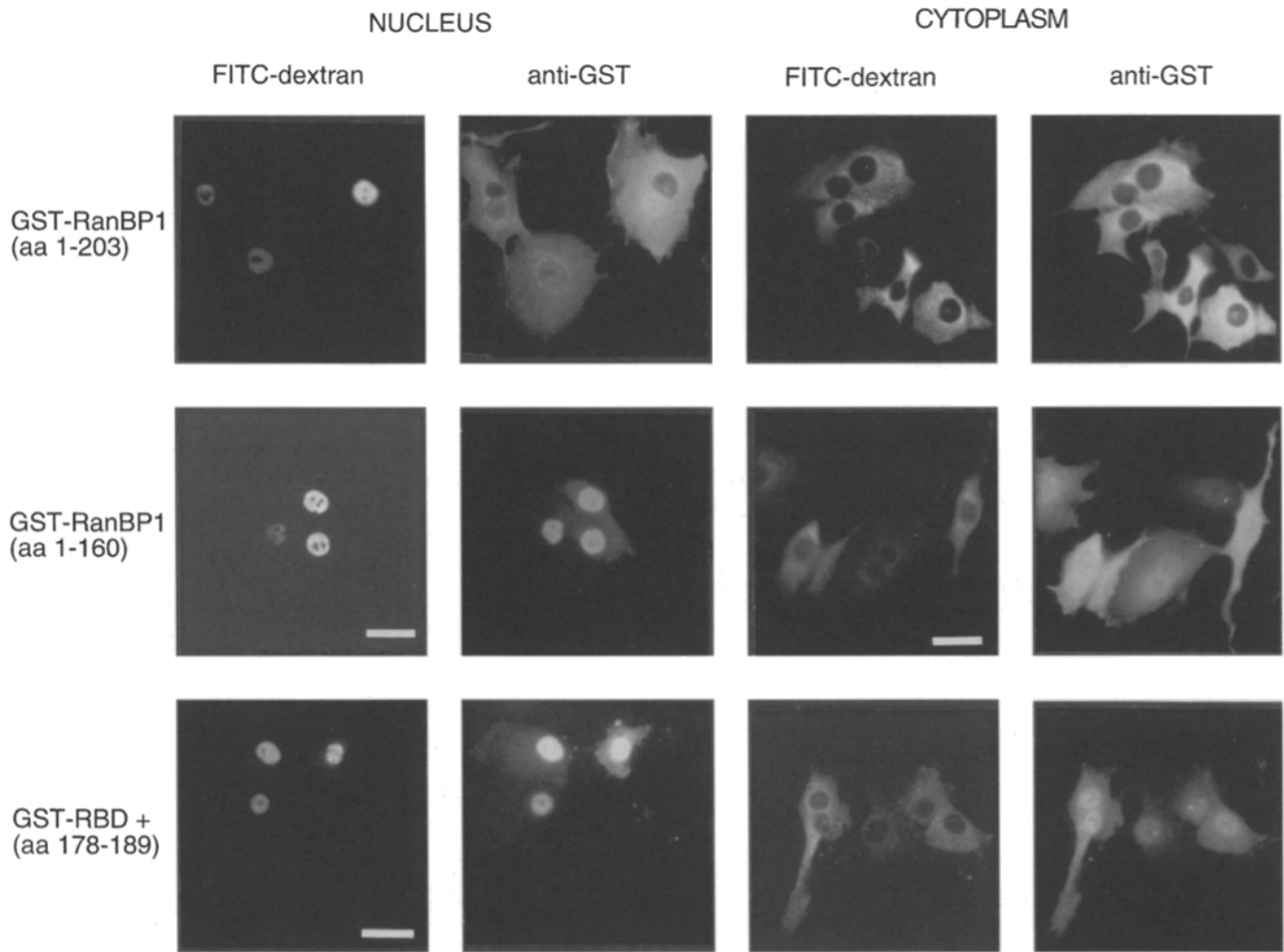
NUCLEUS | CYTOPLASM

FITC-dextran | anti-GST | FITC-dextran | anti-GST

GST-RanBP1 (aa 1-203)

GST-RanBP1 (aa 1-160)

GST-RBD + (aa 178-189)

Figure 9. The RanBP1 COOH terminus contains a nuclear export signal. GST fusion proteins (1.0 mg/ml) were coinjected with FITC-dextran, which marks the injection site in either the nucleus or the cytoplasm. Cells were allowed to recover for 1 h at 37°C, 5% CO_2 before being processed for immunofluoresence with anti-GST primary antibody and a Cy^3-conjugated secondary antibody as described in Materials and Methods. Bar, 25 µm.

al., 1995; Richards et al., 1995), and promote the association of Ran with β-importin/β-karyopherin (Lounsbury et al., 1996). RanBP1 is essential for nuclear protein import and RNA export in *S. cerevisiae* (Schlenstedt et al., 1995b). This study has identified a novel NES in the COOH-terminal domain of mammalian RanBP1, and has demonstrated that the absence of this domain produces a mislocalized RanBP1 that can interfere dominantly with nuclear protein import. The existence of a functional NES in RanBP1 suggests that this protein may provide a link between nuclear protein import and export processes.

The COOH-terminal domain of RanBP1 can function heterologously, localizing a nuclear cyclin B1 in the cytoplasm, and the isolated RanBP1 NES (aa 178–189) will catalyze nuclear export of GST-GFP after microinjection of a GST-GFP-NES fusion protein into the nucleus. However, the isolated NES was unable to drive nuclear export of a functional RBD. In this respect, the RanBP1 NES differs from other known export signals in requiring additional amino acid sequence for the export and cytoplasmic sequestration of its parent protein. This sequence lies

NH$_2$-terminal to the NES and may cooperate with the NES by providing a CRS. There is precedence for two signal sequences being intertwined. The region of hnRNP A1 that contains an NES also contains a nontraditional NLS, and the two signals have not been separated thus far (Michael et al., 1995). In RanBP1, the presence of two signals may be necessary to override the association of the RBD with nuclear Ran.

The observation that the RBD dominantly inhibits nuclear protein import deserves consideration because the biochemistry of the isolated RBD is virtually identical to that of full-length RanBP1 (Beddow et al., 1995; Bischoff et al., 1995; Lounsbury et al., 1996). Ran, an essential component of nuclear protein import, is believed to cycle between the nucleus and the cytoplasm. The presence of the Ran GTPase exchange factor RCC1 in the nucleus implies that nuclear Ran may exist in the GTP bound state (Bischoff and Ponstingl, 1991a,b). The RBD may sequester Ran-GTP in the nucleus, and the consequent depletion of cytoplasmic Ran would arrest nuclear protein import. Therefore, the COOH-terminal CRS/NES domain of RanBP1 may
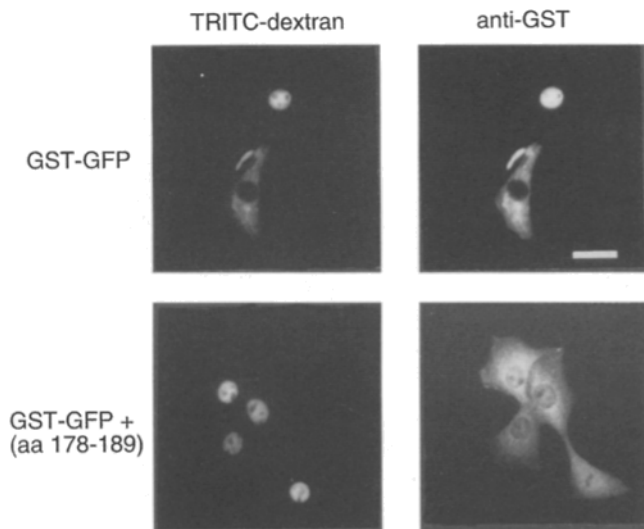
Figure 10. Amino acids 178–189 of RanBP1 encode a functional NES. BHK21 cells were injected with GST-GFP fusion protein (0.5 mg/ml) as described in Materials and Methods. TRITC-dextran was used to mark the injection site. GST-GFP fusions were made with and without RanBP1 amino acids 178–189 at the COOH terminus. After recovering for 2 h at 37°C, 5% CO_2, cells were processed for immunofluorescence with anti-GST primary antibody and a FITC-conjugated secondary antibody. Bar, 25 μm.
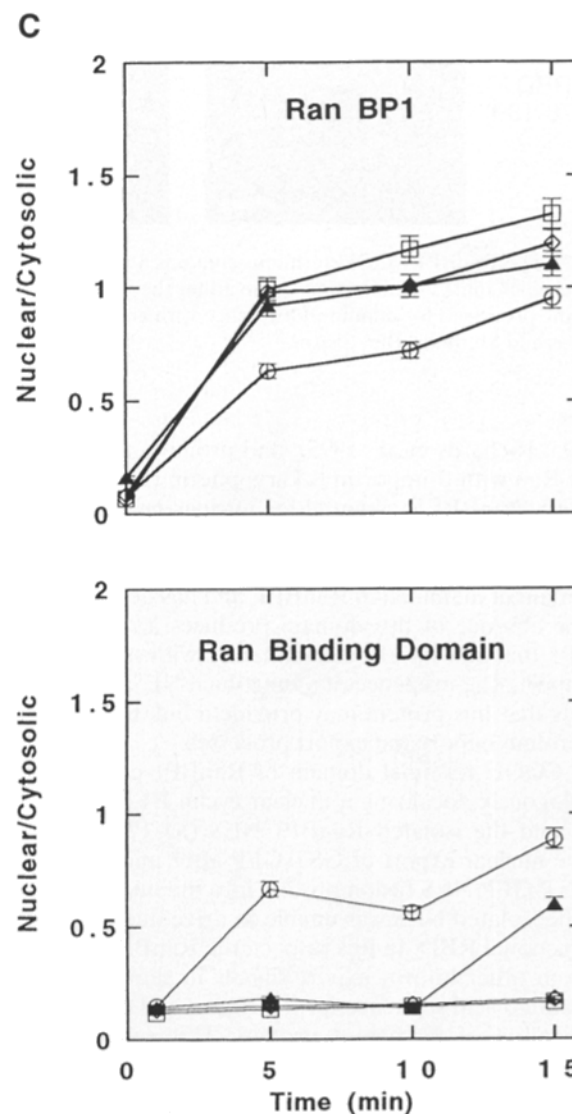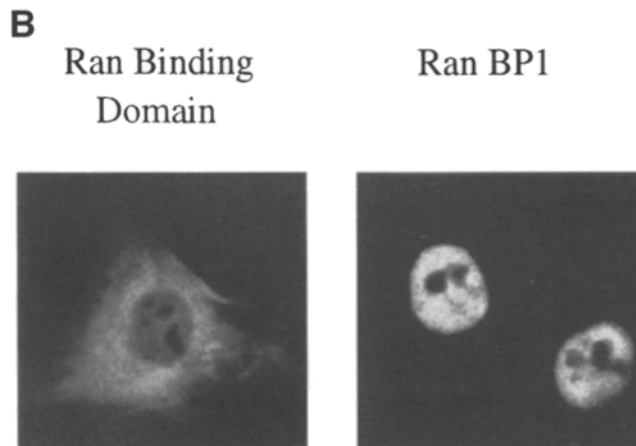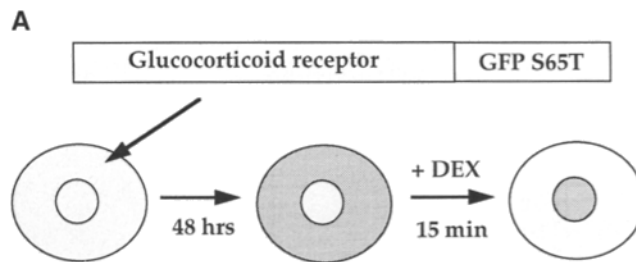
be important for normal function of the protein import machinery.

Similarity to Other NES Sequences

While this work was in progress, NESs were identified in the protein kinase A inhibitor PKI (Wen et al., 1995), HIV-1 Rev (Fischer et al., 1995), and hnRNP A1 (Michael et al., 1995). These three proteins have been shown to shuttle between the nucleus and the cytoplasm, and function to mediate the export of associated molecules.

Two of these proteins are involved in the export of RNA from the nucleus: the HIV-1 Rev protein (Fischer et al., 1995), and heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) (Michael et al., 1995); their export signals bear no resemblance to each other. A third NES has been characterized in PKI (Wen et al., 1995), a factor that es-

Figure 11. Effects of RanBP1 and RBD on nuclear translocation of GR-GFP in living cells. (*A*) Schematic representation of in vivo GR-GFP translocation from the cytoplasm to the nucleus. (*B*) Representative images of GR-GFP in dexamethasone-treated cells cotransfected with HA1-RanBP1 or HA1-RBD. Images were obtained by confocal microscopy using the Bio-Rad MRC-1000 system. (*C*) Time course for nuclear translocation of GR-GFP. BHK21 cells were cotransfected with 2 μg pK7-GR-GFP plus 0 μg (*open circles*), 0.5 μg (*closed triangles*), 1.0 μg (*open diamonds*), or 2.0 μg (*open squares*) pKH³-RanBP1 or pKH³-RBD; pKH³ vector was added to maintain equal total molar quantities of transfected DNA. Cells were treated with 1 μM dexamethasone for indicated times, and the ratio of nuclear to cytoplasmic fluorescence of the GR-GFP was determined as described in Materials and Methods. Bar, 25 μm.
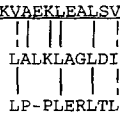
1166

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161-203
mRanBP1: EIEEREKKGPGKNDNAEKVAEKLEALSVREAREEAEEKSEEKQ
                              !! !! ! !
PKI NES:                      LALKLAGLDI
                              !  !  ! !
REV NES:                      LP-PLERLTL
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Figure 12. Amino acid alignment of nuclear export signals. Recently nuclear export signals have been described in protein kinase inhibitor, an inhibitor of protein kinase A (Wen et al., 1995) and HIV-1 Rev (Fischer et al., 1995). Amino acids 178–189 of the RanBP1 COOH terminus are aligned with these NES. Solid lines indicate amino acid identity; dashed lines indicate amino acid similarity.

corts the catalytic subunit of protein kinase A out of the nucleus (Fantozzi et al., 1994).

The region between amino acids 178–189 in RanBP1 shows some similarity to the NES encoded in PKI and in Rev (Fig. 12). Although RanBP1 is not known to shuttle between the nucleus and the cytoplasm, full-length GST-RanBP1 does export from the nucleus, and amino acids 178–189 of RanBP1 are capable of exporting GST-GFP from the nucleus. RanBP1 is most similar to the PKI NES, and these two sequences possess only limited similarity to the Rev NES. However, in all three cases, site-directed mutagenesis has demonstrated a similar functional requirement for specific hydrophobic residues within the export signals (Fig. 7; Bogerd et al., 1995; Stutz et al., 1995; Wen et al., 1995). The TFIIIA protein, which has been implicated in the nuclear export of 5S rRNA (Guddat et al., 1990; Rudt and Pieler, 1996), also possesses a similar pattern of hydrophobic residues (Fischer et al., 1995).

The mechanism by which an NES operates remains unknown. Rab and Rip1p are two factors that have been identified by interaction with the HIV-1 Rev NES domain, and that promote export of the Rev-RNA complex from the nucleus. Rab is a 58-kD nucleoplasmic protein (Bogerd et al., 1995), and Rip1p is a 42-kD protein that is concentrated at the nuclear pore (Stutz et al., 1995). Both proteins contain a reiterated FG dipeptide motif that is also present in a subset of nucleoporins (Gerace, 1995).

To begin to address whether these NES sequences operate via a common mechanism, we tested in vitro association between RanBP1 and the Rev-binding protein, Rab (Bogerd et al., 1995). Using coprecipitation of HA1-Rab and endogenous RanBP1 as detected by Ran-GTP overlay assay, and association of HA1-Rab with GST-RanBP1 as detected by anti-HA1 Western blot, we were unable to detect any specific association between these two proteins (data not shown). These data suggest that the export signals and export mechanisms for RanBP1 and Rev may differ.

Evidence for a Cytoplasmic Retention Signal in RanBP1

Although amino acid residues 178–189 of RanBP1 can function as an NES in exporting GST-GFP, this region is not sufficient to relocalize or retain a functional RBD in the cytoplasm. Therefore, it is likely that other factors are involved in excluding full-length RanBP1 from the nucleus. One possibility is the presence of a CRS residing within amino acids 161–189 at the COOH terminus of RanBP1.

The existence of a CRS has been suggested for cyclin B (Pines and Hunter, 1994). In their analysis of regions responsible for the localization of the human cyclins B1 and B2, Pines and Hunter were able to delineate portions of these proteins necessary for the cyclin cytoplasmic retention. When these regions were aligned with the COOH-terminal region of RanBP1, no obvious similarity was detected, but there may be different retention signals for different categories of cytoplasmic proteins.

Role of the NES/CRS in RanBP1 Function

Why does RanBP1 require an NES/CRS? One obvious explanation is that RanBP1 is small enough to passively diffuse into the nucleus, and in the absence of an export mechanism it would sequester nuclear Ran and inhibit protein import. However, by analogy with the other known NES-containing proteins that associate with export cargo, we speculate that RanBP1 may function to mediate the export of GTP-bound Ran. As discussed above, Ran is primarily nuclear, and the Ran exchange factor is exclusively nuclear. However, cytosolic Ran is essential for nuclear protein import, and the docking at nuclear pores requires Ran in the GTP-bound state (Melchior et al., 1995b). A mechanism for the export of Ran-GTP to the cytoplasm therefore appears to be a necessary component of the Ran cycle, and an NES on a Ran-GTP binding protein may provide such a mechanism.

The RanBP1 NES/CRS may also play a role during the mammalian mitotic cell cycle. When the nuclear envelope breaks down at the beginning of M phase in mitosis, the soluble nuclear and cytoplasmic components comingle. Upon reformation of the nuclear membrane, the RanBP1 must be exported from the nascent nucleus while the majority of the cellular Ran accumulates in the nucleus. The NES/CRS of RanBP1 may ensure that this spatial separation occurs. The cytoplasmic RanBP1 may also provide a pool of cytoplasmic Ran-GTP necessary for restarting the nuclear protein import process after the reformation of the nuclear membrane. The *S. cerevisiae* RanBP1 homologue is cytoplasmic (Schlenstedt et al., 1995b) but does not contain a region resembling the RanBP1 or PKI NES. If the RanBP1 has a mitotic role, the closed mitosis in yeast may alleviate the need for an NES.

Whatever the cellular role of RanBP1, it is apparent that it contains a nuclear export signal that is necessary but not sufficient for the cytoplasmic location of this protein. Further analysis of this region will allow us to determine if this sequence is involved in a general mechanism for the nuclear export and localization of small cytoplasmic proteins, and what other proteins interact with this region of RanBP1.

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