NXT1 Is Necessary for the Terminal Step of Crm1-mediated Nuclear Export

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Abstract. Soluble factors are required to mediate nuclear export of protein and RNA through the nuclear pore complex (NPC). These soluble factors include receptors that bind directly to the transport substrate and regulators that determine the assembly state of receptor–substrate complexes. We recently reported the identification of NXT1, an NTF2-related export factor that stimulates nuclear protein export in permeabilized cells and undergoes nucleocytoplasmic shuttling in vivo (Black, B.E., L. Lévesque, J.M. Holaska, T.C. Wood, and B.M. Paschal. 1999. *Mol. Cell. Biol.* 19:8616–8624). Here, we describe the molecular characterization of NXT1 in the context of the Crm1-dependent export pathway. We find that NXT1 binds directly to Crm1, and that the interaction is sensitive to the presence of Ran-GTP. Moreover, mutations in NXT1 that reduce binding to Crm1 inhibit the activity of NXT1 in nuclear export assays. We show that recombinant Crm1 and Ran are sufficient to reconstitute nuclear translocation of a Rev reporter protein from the nucleolus to an antibody accessible site on the cytoplasmic side of the NPC. Further progress on the export pathway, including the terminal step of Crm1 and Rev reporter protein release, requires NXT1. We propose that NXT1 engages with the export complex in the nucleoplasm, and that it facilitates delivery of the export complex to a site on the cytoplasmic side of NPC where the receptor and substrate are released into the cytoplasm.

Key words: nuclear transport • NES • Crm1 • Ran • NXT1

Introduction

Protein and RNA transport across the nuclear envelope (NE)[1] occurs through specialized channels termed nuclear pore complexes (NPCs) (Gorlich and Kutay, 1999; Nakielny and Dreyfuss, 1999). The NPC is estimated to contain >50 different proteins, which assemble into a supramolecular structure (~125 mD) that spans the double membrane of the NE (Stoffler et al., 1999; Wente, 2000). The NPC contains a central channel that mediates facilitated import and export of a large variety of transport substrates. The specificity of transport, that is to say whether a macromolecule undergoes import or export, generally requires a cis-acting nuclear localization signal (NLS) or a nuclear export signal (NES). These signals are recognized by transport receptors, which mediate interactions with the NPC and facilitate translocation of the macromolecule to the appropriate compartment.

Most of the receptors for import and export described to date are members of the importin-β (also called karyopherin-β) superfamily of proteins. This includes Crm1, the nuclear export receptor for NES-containing proteins, such as protein kinase inhibitor (PKI) (Wen et al., 1995; Fornerod et al., 1997; Stade et al., 1997). In the nucleus, Ran-GTP stabilizes Crm1 binding to NES-containing proteins, resulting in the formation of a trimeric export complex (Fornerod et al., 1997). The physical basis of export complex translocation through the NPC, including the mechanisms that influence the direction of export complex movement through the NPC, have not been defined. There is also limited data addressing the terminal step of export, which refers to the posttranslocation events that result in disassembly of the export complex, and in the case of Crm1, release of its NES-containing cargo into the cyto-

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[1]*Abbreviations used in this paper:* CTE, constitutive transport element; GFP, green fluorescent protein; GR, glucocorticoid receptor; GST, glutathione-*S*-transferase; LMB, leptomycin B; NE, nuclear envelope; NES, nuclear export signal; NLS, nuclear localization signal; NPC, nuclear pore complex; PKI, protein kinase inhibitor; RRE, Rev response element; STV, streptavidin.

plasm. One key component in the terminal step of export is Ran-GTP, which has the capacity to serve as a molecular switch to terminate both NES binding to Crm1, and Crm1 binding to the NPC (Fornerod et al., 1997; Kehlenbach et al., 1999). The terminal step of export could occur when a protein binds Ran-GTP and dissociates it from the export complex. The Ran-binding protein RanBP1, or RanBP1-like domains in the nucleoporin RanBP2, are candidates for promoting the dissociation reaction in this manner (Kehlenbach et al., 1999). Alternatively, RanGAP could activate the Ran GTPase in the context of the export complex, perhaps using RanBP1 as a cofactor, and convert GTP to GDP (Bischoff et al., 1995; Askjaer et al., 1999; Paraskeva et al., 1999). Because of its extremely low affinity for Crm1, Ran-GDP would rapidly dissociate from the complex, resulting in NES release from Crm1. RanGAP could also function in the dissociation-based mechanism by activating the GTPase activity of Ran-GTP that has already dissociated from the export complex. In both cases, RanGAP-dependent conversion of Ran-GTP to Ran-GDP would make export complex reassembly unfavored, making the disassembly reaction essentially irreversible. These models for terminating export are consistent with the electron microscopic immunolocalization of RanGAP and RanBP2 on the cytoplasmic side of the NPC (Matunis et al., 1996; Mahajan et al., 1997), as well as the cytoplasmic distribution of RanBP1 (Richards et al., 1996).

The mechanisms of nuclear import and export are of interest because of their importance to basic cell physiology and their relevance to the life cycles of medically important viruses. Retroviruses, such as HIV-1, require specific interactions with the host cell machinery for both nuclear import and export of viral proteins and nucleic acids (Stevenson, 2000). Integration of the viral genome into the DNA of nondividing cells requires translocation of the preintegration complex through the NPC in a mechanism that involves importin-β as the import receptor. Transcription of the viral genome generates an ~13-kb RNA, which is exported to the cytoplasm and used as the template for protein synthesis and virion production (Cullen, 2000). Nuclear export of HIV-1 RNA depends on a cis-acting signal within the RNA, which is termed the Rev response element (RRE) (Malim et al., 1989). The RRE provides the binding site for Rev, an HIV-1 protein whose leucine-rich NES is, in turn, recognized by the host export machinery (Fischer et al., 1995). This occurs when the export receptor Crm1 binds to the NES of Rev, an interaction that is stabilized by Ran-GTP. Current models suggest that a complex consisting of RRE RNA, Rev, Crm1, and Ran-GTP assembles in the nucleoplasm and undergoes translocation through the NPC (Gorlich and Kutay, 1999; Nakielny and Dreyfuss, 1999). In the absence of an efficient pathway for nuclear export, HIV-1 RNA is subject to nuclear retention and splicing because it contains introns (Sodroski et al., 1986). Thus, nuclear export of full-length genomic RNA is necessary for viral replication.

In previous work, we identified a protein we named NXT1 for NTF2-related export protein 1 (Black et al., 1999). The evolutionary relatedness of NXT1 and NTF2 (26% identity) suggests these proteins should have related functions in the cell. We found that NTF2 binds Ran-GDP and functions in nuclear import (Moore and Blobel, 1994; Paschal and Gerace, 1995; Paschal et al., 1996), and that NXT1 binds Ran-GTP and functions in nuclear export (Black et al., 1999). Both NXT1 and NTF2 are nuclear proteins, and NXT1 shuttles between the nucleus and cytoplasm. One striking difference is that the steady-state distribution of NXT1 includes the NPC. The NPC localization of NXT1 persists after digitonin permeabilization of the plasma membrane, a condition that releases many soluble proteins from the cell. Evidence that NXT1 is an export factor was obtained by showing that the recombinant protein stimulates nuclear export of PKI in permeabilized cells (Black et al., 1999). NXT1-dependent export of PKI required the addition of cytosol and could be blocked by the Crm1-specific inhibitor leptomycin B (LMB) (Wolff et al., 1997). These findings, together with more recent data showing that NXT1 can stimulate nuclear export of U1 snRNA in permeabilized cells (Ossareh-Nazari et al., 2000), led us to conclude that NXT1 functions on an export pathway for which Crm1 is the receptor.

NXT1, which has been referred to as p15, has also been described as a cofactor for a viral RNA export pathway for which a protein termed TAP is the receptor (Katahira et al., 1999). TAP can bind directly to the constitutive transport element (CTE) of transcripts encoded by the Mason-Pfizer monkey virus (Hammarskjöld, 1997), and it can stimulate nuclear export of the RNA in oocytes (Gruter et al., 1998). Although NXT1 binds directly to TAP (Katahira et al., 1999; Lévesque, L., and B.M. Paschal, unpublished observations), there is no data addressing how this interaction regulates CTE RNA export. The host cell function of the NXT1–TAP interaction may involve nuclear export of mRNA. This prediction is based on the relatedness of TAP to the mRNA export factor Mex67p (23% identity, expectation value 10^{-11}), and the fact that coexpression of NXT1 and TAP can rescue the viability of a *mex67* mutant in *Saccharomyces cerevisiae* (Katahira et al., 1999).

Here, we have characterized the molecular function of NXT1 in Crm1-mediated nuclear export. We have used a cell-based assay and recombinant factors to reconstitute nuclear export of a Rev reporter protein. We find that Crm1 and Ran are sufficient to reconstitute Rev transport from the nucleolus to the cytoplasmic side of the NPC. This likely reflects the accumulation or arrest of the export complex at an intermediate step in the pathway. We demonstrate that NXT1 is required for progression through the terminal step in the nuclear export pathway, resulting in the release of Crm1 and Rev from the cytoplasm side of the NPC. The terminal step also requires RanBP1 as a cofactor, which may reflect RanBP1- and RanGAP-dependent conversion of Ran-GTP to Ran-GDP as part of the release mechanism. We show that NXT1 binds directly to Crm1, and a point mutation in NXT1 that reduces Crm1 binding also reduces its export activity. Our results indicate that NXT1 is a cofactor that facilitates the terminal step in Crm1-dependent export.

Materials and Methods

Rev Export Assay

Rev export was monitored using a cell line (RGG2.2) (Love et al., 1998) expressing Rev fused to the ligand-binding domain of the glucocorticoid receptor (GR) and green fluorescent protein (GFP). In brief, nuclear accumulation of the Rev–GR–GFP is stimulated by the addition of 1 μM dexamethasone for 30 min to RGG2.2 cells growing on coverslips in DME

containing 10% newborn calf serum. Cells are permeabilized with digitonin (0.005%) for 5.5 min. This was followed by a 4-min incubation in transport buffer (20 mM Hepes, pH 7.4, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA) supplemented with 300 mM NaCl to release NXT1 from the NPC. Standard export reactions (50 μl) were carried out at 30°C for 30 min using the combinations of recombinant proteins stated in the figure legends. A protease inhibitor cocktail, which consisted of leupeptin and pepstatin (each at 1 μg/ml), as well as 2 mM DTT, was included in all transport reactions. HeLa cell cytosol was prepared, as described previously (Holaska and Paschal, 1998), and used at 3 mg/ml as a positive control for nuclear export. Phenyl-Sepharose treatment of cytosol to deplete Crm1 was performed as described previously (Holaska and Paschal, 1998). The order of addition experiments (see Fig. 4) are two-step export reactions that involve a standard export reaction, a wash step, and an additional 30-min incubation with the indicated factors. Cells from both standard and two-step export reactions were fixed with formaldehyde, stained with DAPI, and mounted using Vectashield medium (Vector Laboratories). Digital images were captured by a charged-coupled device camera (Hamamatsu ORCA) mounted on a Nikon Microphot-SA microscope, using Openlab (version 2.0.6) software. Figures were assembled using Adobe Photoshop® (version 5.5) and Freehand (version 8.0). Images were captured with the same exposure times within an experiment. All images shown are representative of the results from multiple experiments.

Anti-GFP Detection Assay

For experiments involving antibody-based detection of Rev–GR–GFP, cells were fixed in 2% formaldehyde for 10–15 min. This relatively light fixation maintains NE integrity. Samples were incubated in a blocking solution containing PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) supplemented with 2% BSA and 2% newborn calf serum for 45 min. The affinity-purified anti-GFP rabbit polyclonal antibody (provided by Pamela Silver, Harvard University, Cambridge, MA) (Seedorf et al., 1999) was used at 1:20,000 for detection of Rev–GR–GFP. To visualize NPCs, a pan-nucleoporin mAb (RL1) (provided by Larry Gerace, The Scripps Research Institute, La Jolla, CA) (Snow et al., 1987) was used at 1:100. To visualize Crm1 in Rev–GR–GFP export assays, cells were fixed lightly and incubated with an anti-Crm1 rabbit polyclonal antibody (provided by Ralph Kehlenbach, The Scripps Research Institute, La Jolla, CA) (Kehlenbach et al., 1998) diluted 1:1,000. Goat anti–rabbit Cy3-conjugated secondary antibody and goat anti–mouse Cy3-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories.

PKI Export Assay

The PKI export assays were performed in suspension-culture HeLa cells, as described previously (Holaska and Paschal, 1998). In brief, nuclear import of FITC–streptavidin (STV)–NLS in digitonin permeabilized cells is mediated by the phenyl-Sepharose–treated cytosol, which is depleted of Crm1, as assayed by immunoblotting (Holaska and Paschal, 1998). Biotinylated PKI enters the nucleus via diffusion and binds the FITC–STV–NLS. Export reactions were performed using the indicated export factors, the samples were fixed, and mounted on slides. Nuclear export in this assay is not dependent on the addition of recombinant RanBP1. This is likely due to the fact that the cytosol used to drive the import of FITC–STV–NLS contains RanBP1, and it is not released efficiently during the wash step between the import and export steps of the assay. Nuclear export was quantitated by measuring the reduction of nuclear fluorescence that occurs upon the addition of export factors (Holaska and Paschal, 1998). The reduction in nuclear fluorescence in our assay is a result of nuclear export and not degradation of FITC–STV–NLS (Holaska and Paschal, 1998; data not shown).

Recombinant Proteins

The export factors used to reconstitute nuclear export were expressed as recombinant proteins in bacteria and stored at −80°C as single-use aliquots. The expression and purification of His-tagged Ran (Gorlich et al., 1994), glutathione-*S*-transferase (GST)–tagged Ran (Richards et al., 1995), His-tagged Crm1 (Englmeier et al., 1999), GST-tagged RanBP1 (either intact or thrombin-cleaved) (Richards et al., 1995), and biotinylated PKI (Holaska and Paschal, 1998) has all been described. Wild-type NXT1 (sequence data available from GenBank/EMBL/DDBJ under accession no. AF156958) expression and purification from the inclusion body fraction has been described previously (Black et al., 1999). Point mutations in the NXT1 open reading frame were generated in the bacterial expression vector using the QuickChange site-directed mutagenesis system (Stratagene). The COOH-terminal truncation was generated in a PCR reaction that amplified the NXT1 open reading frame, with a stop codon engineered to remove the final four codons. The protocol used for expression and purification of the wild-type NXT1 protein was used for the mutant NXT1 proteins.

Protein-binding Assays

Solid-phase Ran-binding assays have been described previously (Black et al., 1999). In brief, recombinant proteins were adsorbed to microtiter wells (5 μg NXT1 added/well, each well with a capacity of ~20 ng), which was followed by extensive wash and blocking steps (Black et al., 1999). Ran, preloaded with [γ-^{32}P]GTP or [α-^{32}P]GTP, was incubated in wells containing the indicated proteins immobilized in high-binding 96-well plates (Corning). Similarly, [^{35}S]Crm1 (Ossareh-Nazari and Dargemont, 1999), expressed in reticulocyte lysate, was incubated in wells containing the indicated immobilized proteins. The radiolabeled proteins that bound to the wells were eluted with SDS and quantitated by scintillation counting. Ran or Crm1 binding to BSA-coated wells was designated background and subtracted from the wells containing the immobilized recombinant proteins in both radiolabel and ELISA detection methods.

Recombinant Crm1 binding in the solid-phase assays was measured by ELISA (Delphin et al., 1997) using an anti-Crm1 antibody. After the binding reaction, wells were washed 3×, and the anti-Crm1 antibody (1:1,000) was incubated in 0.5× transport buffer containing 0.2% Tween 20 and 1% BSA for 2 h at room temperature. Wells were washed 5× and goat anti–rabbit horseradish peroxidase–coupled secondary antibody (Pierce Chemical Co.) was incubated in the same buffer for 1 h at room temperature. Horseradish peroxidase activity was measured, using 2,2′ azino-di-(3-ethylbenzthiozoline sulfonic acid) as a colorimetric substrate, on a microplate reader (ELX800; Bio-Tek Instruments, Inc.) to measure the absorbance at 410 nm. NXT1 binding in solid-phase assays was analyzed by immunoblot using a rabbit polyclonal antibody raised against a peptide (CGTVKFEGNKQRDFNQN) that corresponds to an internal sequence in mouse NXT1. All binding assays were performed in duplicate and the values shown represent the mean ± SD.

RNA Export Assays

U1 snRNA, mRNA, and tRNA export assays in permeabilized HeLa cells have been described previously (Ossareh-Nazari et al., 2000). After the export reactions, samples were centrifuged at 210 *g* for 5 min to separate nuclear and supernatant fractions. Radiolabeled RNA was visualized after separation on an 8% acrylamide gel containing 7 M urea. The level of export was quantitated using the Bioprint acquisition system and Bioprofil software. The values for RNA export correspond to the ratio of RNA in the supernatant fraction to the sum of RNA present in the nuclear and supernatant fractions. Quantitation of U1 snRNA and tRNA export is from three independent experiments. Quantitation for mRNA export is from two independent experiments.

Results

NXT1 Stimulates Rev Export

NXT1 is a Ran-GTP–binding protein predicted to function on Crm1-dependent export pathways, based on two observations. First, recombinant NXT1 stimulates nuclear export of the Crm1-transport substrates PKI (Black et al., 1999) and U1 snRNA (Ossareh-Nazari et al., 2000) in permeabilized cells supplemented with cytosol. Second, NXT1-stimulated export of PKI in permeabilized cells is blocked by the addition of LMB (Black et al., 1999), a highly specific inhibitor of Crm1 function. We set out to characterize how NXT1 may regulate Crm1-dependent export using recombinant factors and permeabilized cell assays that reconstitute nuclear export of NES-containing fluorescent reporter proteins.

We used a cell line that expresses the NES-containing protein Rev fused to the ligand-binding domain of GR and GFP, which undergoes nuclear import after dexametha-

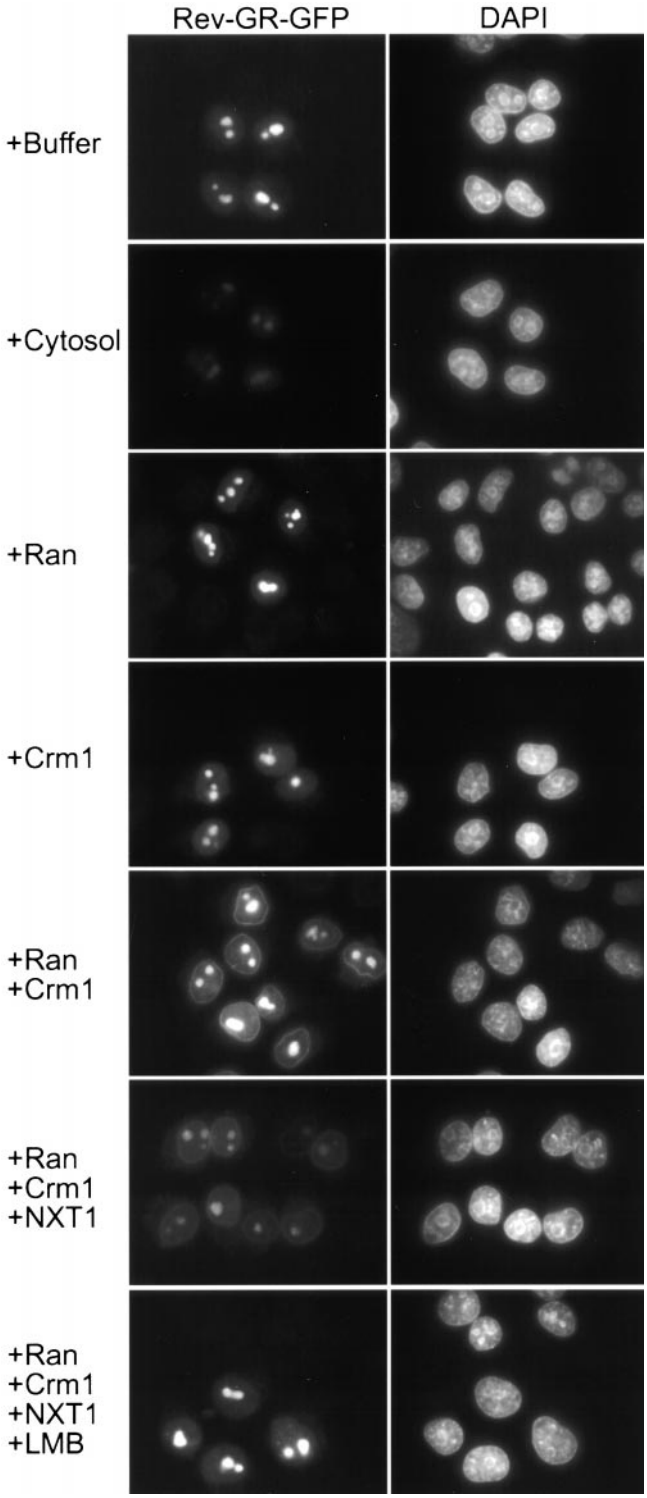


Figure 1. NXT1 cooperates with Crm1 and Ran to mediate the nuclear export of Rev. Rev export assays were performed in permeabilized cells expressing Rev–GR–GFP (Love et al., 1998). Ran (1.5 μM) and Crm1 (16 nM), when present in the same reaction, promote the accumulation of Rev–GR–GFP export substrate at the NE (+Ran+Crm1). This accumulation was observed when either recombinant His-tagged Crm1 (shown here) or Crm1 purified from HeLa cytosol (data not shown) was used in reactions containing Ran. NXT1 (17 μM) stimulated nuclear export when both Ran and Crm1 were present in the reaction (+Ran+Crm1+NXT1), and this export was sensitive to the Crm1-specific inhibitor LMB (500 nM) (+Ran+Crm1+NXT1+LMB). NXT1 had no effect in export reactions supplemented with Ran or Crm1 separately (data not shown). The fields shown were captured using a 60× oil immersion objective, and the NXT1 stimulation of Rev–GR–GFP export (+Ran+Crm1+NXT1) is representative of the effect observed in 25 separate experiments.

sone addition (Love et al., 1998). The Rev–GR–GFP fusion concentrates in nucleoli, and after digitonin permeabilization, undergoes cytosol-dependent nuclear export (Fig. 1; and Love et al., 1998). We observed that Rev–GR–GFP translocation from nucleoli to the NPC, indicated by the accumulation of fluorescence at the NE, requires the addition of Ran and Crm1 (Fig. 1). We found that NXT1 stimulates Ran and Crm1-dependent export, resulting in a marked reduction in both nucleolar- and NE-associated fluorescence. NXT1-stimulated export of Rev–GR–GFP was blocked by LMB (Fig. 1). This is consistent with our previous data showing that NXT1 stimulation of PKI export can be blocked by LMB (Black et al., 1999). A low level of Rev–GR–GFP was detected at the NE in the reaction containing Ran, Crm1, and NXT1, which was absent in the reaction containing cytosol (Fig. 1). This suggests that additional factors, such as RanBP1, may be required for highly efficient release of NES substrate from the NPC in permeabilized cells (see below). In summary, these data indicate that NXT1, Crm1, and Ran-GTP function cooperatively to mediate nuclear export of the NES substrate Rev–GR–GFP.

NXT1 Facilitates Late Events in the NES Export Pathway

The Crm1- and Ran-dependent accumulation of Rev–GR–GFP at the NE is likely to represent its association with the NPC. We confirmed this by showing that the Rev–GR–GFP signal is coincident with the NPC (Fig. 2, NE Permeabilized, α-NPC), using the pan-nucleoporin antibody RL1 (Snow et al., 1987). To determine whether Rev–GR–GFP accumulation occurs on the nuclear or cytoplasmic side of the NPC, we used an antibody to GFP (Seedorf et al., 1999) and stained the cells after permeabilization with digitonin. Since the NE remains intact under this condition, the cytoplasmic side of the NPC is accessible to the antibody, whereas the nucleoplasmic side is not. Using the anti-GFP detection assay, we found that Rev–GR–GFP accumulates on the cytoplasmic side of the NPC in the presence of Crm1 and Ran (Fig. 2, NE Intact, α-GFP), but not in the absence of these factors (see below). Pretreatment of cells with 1% Triton X-100 resulted in permeabilization of the NE and detection of GFP in both compartments (Fig. 2, NE Permeabilized). Thus, a complex consisting minimally of Crm1, Ran-GTP, and Rev–GR–GFP is delivered to the cytoplasmic side of the NPC in our permeabilized cell assay, and it can be revealed by antibody detection.

We used our anti-GFP detection assay to define the biochemical requirements for Rev–GR–GFP release from the cytoplasmic face of the NPC, which is likely to represent the terminal step in nuclear export. We found that whereas Ran and Crm1 promote Rev–GR–GFP delivery to the cytoplas-

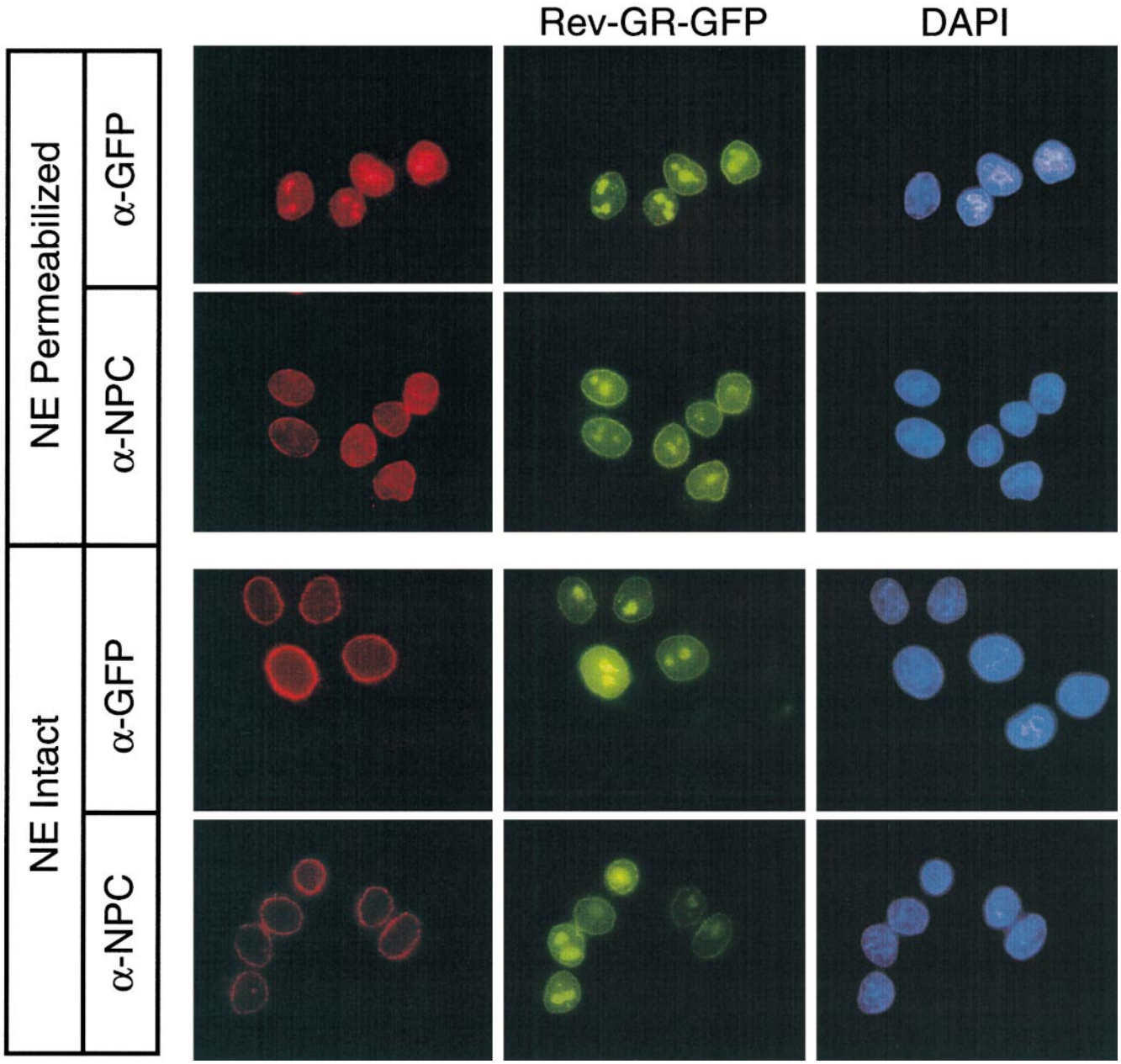


Figure 2. Rev–GR–GFP accumulates at the cytoplasmic face of the NPC in a reaction containing Crm1 and Ran. Export reactions were performed in the presence of Crm1 (16 nM) and Ran (1.5 μM). NPCs were detected with a pan-nucleoporin antibody (RL1) (Snow et al., 1987) to reveal punctate staining at the NE. In cells that were treated with detergent (1% Triton X-100), the anti-GFP antibody has access to the nucleoplasm, and the staining pattern is coincident with the GFP signal observed in the FITC channel. In cells that were not treated with detergent, NE integrity is maintained. Under this latter condition, the anti-GFP antibody cannot enter the nucleus, though robust punctate staining is present on the NE of cells expressing Rev–GR–GFP. Cells not expressing Rev–GR–GFP do not show any anti-GFP staining, illustrating the specificity of the antibody and conditions used in this assay.

mic face of the NPC, including NXT1 in this reaction significantly reduced the level of cytoplasm-exposed Rev–GR–GFP (Fig. 3, +Ran+Crm1+NXT1). We also examined the ability of RanBP1 to promote Rev–GR–GFP release from the NPC, since it has been shown previously to act as a cofactor for NES cargo release (Kehlenbach et al., 1999). Including recombinant RanBP1 in the export reaction with Ran-GTP and Crm1 had little effect on the level of Rev–GR–GFP detected on the cytoplasmic side of the NPC, and RanBP1 did not appear to stimulate nuclear export of Rev–GR–GFP, as judged by the bright nucleolar fluorescence in cells at the end of the export reaction (Fig. 3, +Ran+Crm1+RanBP1). However, we found that including both NXT1 and RanBP1 in the reaction promoted the complete release of Rev–GR–GFP from the cytoplasmic side of the NPC, as well as a dramatic reduction in nucleolar fluorescence (Fig. 3, +Ran+Crm1+NXT1+RanBP1). Our finding that Ran, Crm1, NXT1, and RanBP1 are sufficient for Rev–GR–GFP delivery to, and release from, the cytoplasmic side of the NPC suggests these are the minimal sol-

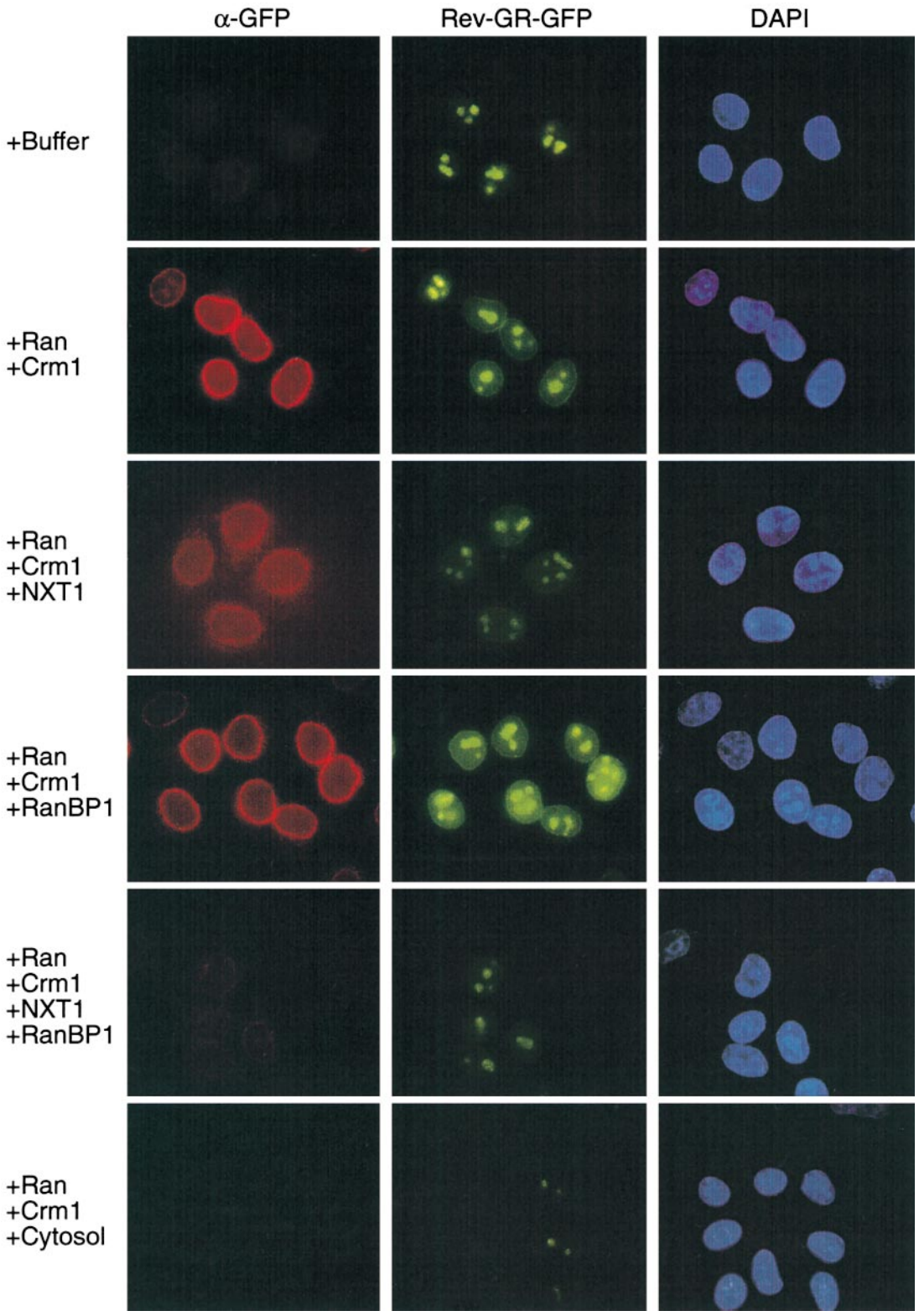


Figure 3. NXT1 facilitates the release of Rev–GR–GFP from the NPC. Rev export reactions were performed in the presence of the indicated factors. Rev–GR–GFP on the cytoplasmic face of the NPC was visualized using the anti-GFP antibody under conditions where the integrity of the NE is maintained. The addition of NXT1 (17 μM) to a reaction containing Ran (1.5 μM) and Crm1 (16 nM) caused a marked decrease in Rev–GR–GFP on the cytoplasmic face of the NPC (+Ran+Crm1+NXT1). RanBP1 (1.7 μM) had a modest effect when added alone to a reaction containing Ran and Crm1 (+Ran+Crm1+RanBP1), but completely released the Rev–GR–GFP remaining on the cytoplasmic face of the NPC when added to a reaction containing Ran, Crm1, and NXT1 (+Ran+Crm1+NXT1+RanBP1). Cytosol, when added to the Ran- and Crm1-containing reaction, also releases Rev–GR–GFP from the cytoplasmic face of the NPC (+Ran+Crm1+Cytosol). The GFP signal is shown for each sample to illustrate the level of export that occurs under each of these conditions.

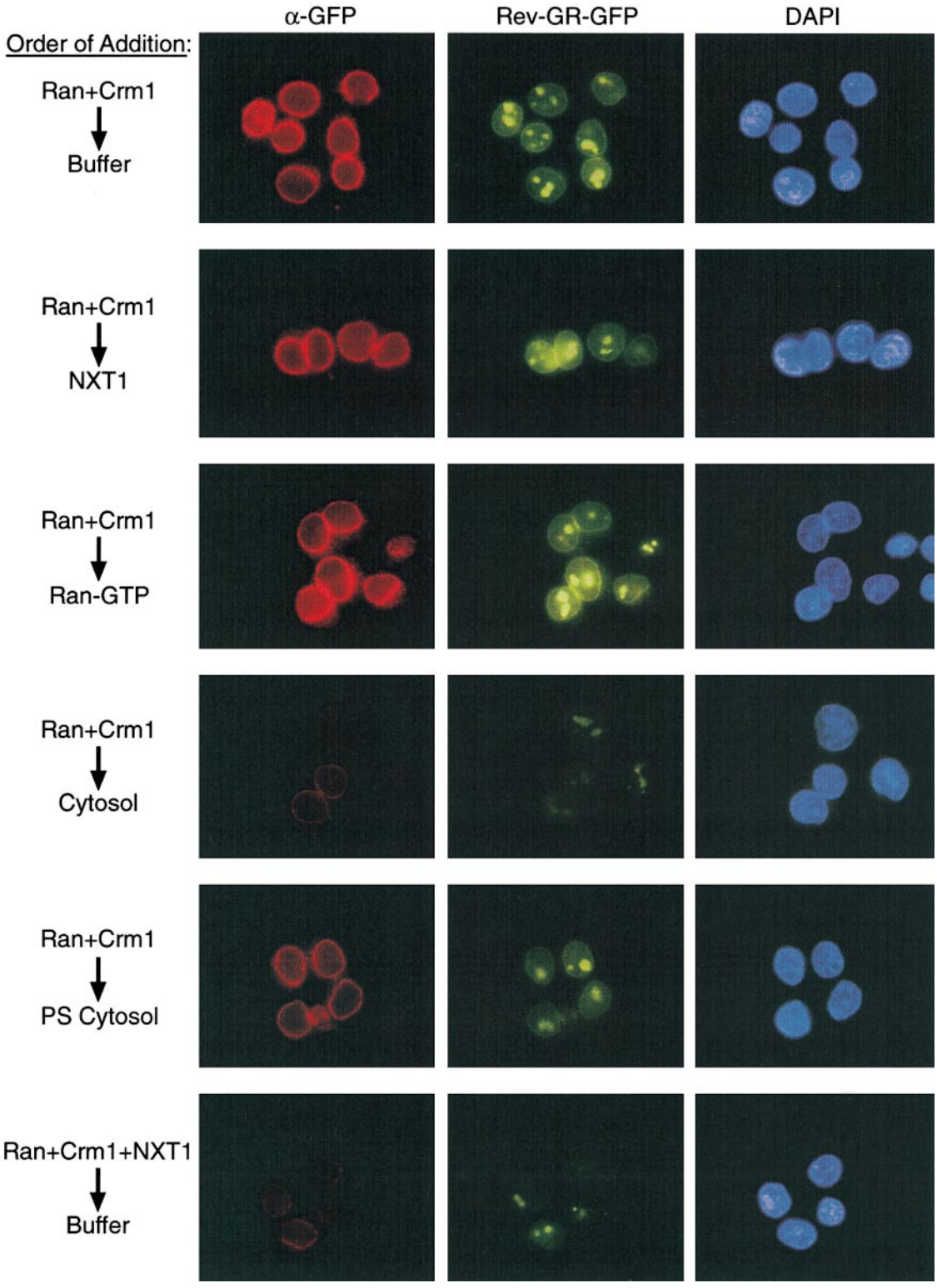


Figure 4. Order of addition experiment indicating the requirement for Ran, Crm1, and NXT1 early in the export pathway. Rev export reactions were performed in two steps. The first step was a standard export reaction using the proteins indicated (Ran+Crm1 or Ran+Crm1+NXT1). The samples were then washed twice, and incubated during the second step with buffer or the proteins indicated. Ran was used at 1.5 μM, Crm1 was used at 16 nM, and NXT1 was used at 17 μM. NXT1, added in a second step, promotes little Rev–GR–GFP release. In contrast, when NXT1 is included in the first step, we observe nearly complete release of Rev–GR–GFP. When Ran (1.5 μM), preloaded with GTP, is added in a second step, it does not release Rev–GR–GFP from the cytoplasmic face of the NPC. However, Rev–GR–GFP can be released by the addition of cytosol (3 mg/ml). Phenyl-Sepharose–treated cytosol (PS cytosol), which is depleted of Crm1 (Holaska and Paschal, 1998), is less active than untreated cytosol in releasing Rev–GR–GFP from the cytoplasmic face of the NPC, suggesting a role for Crm1 in this reaction. Note that RanBP1 (1.7 μM) was included in the first and second steps of all reactions.

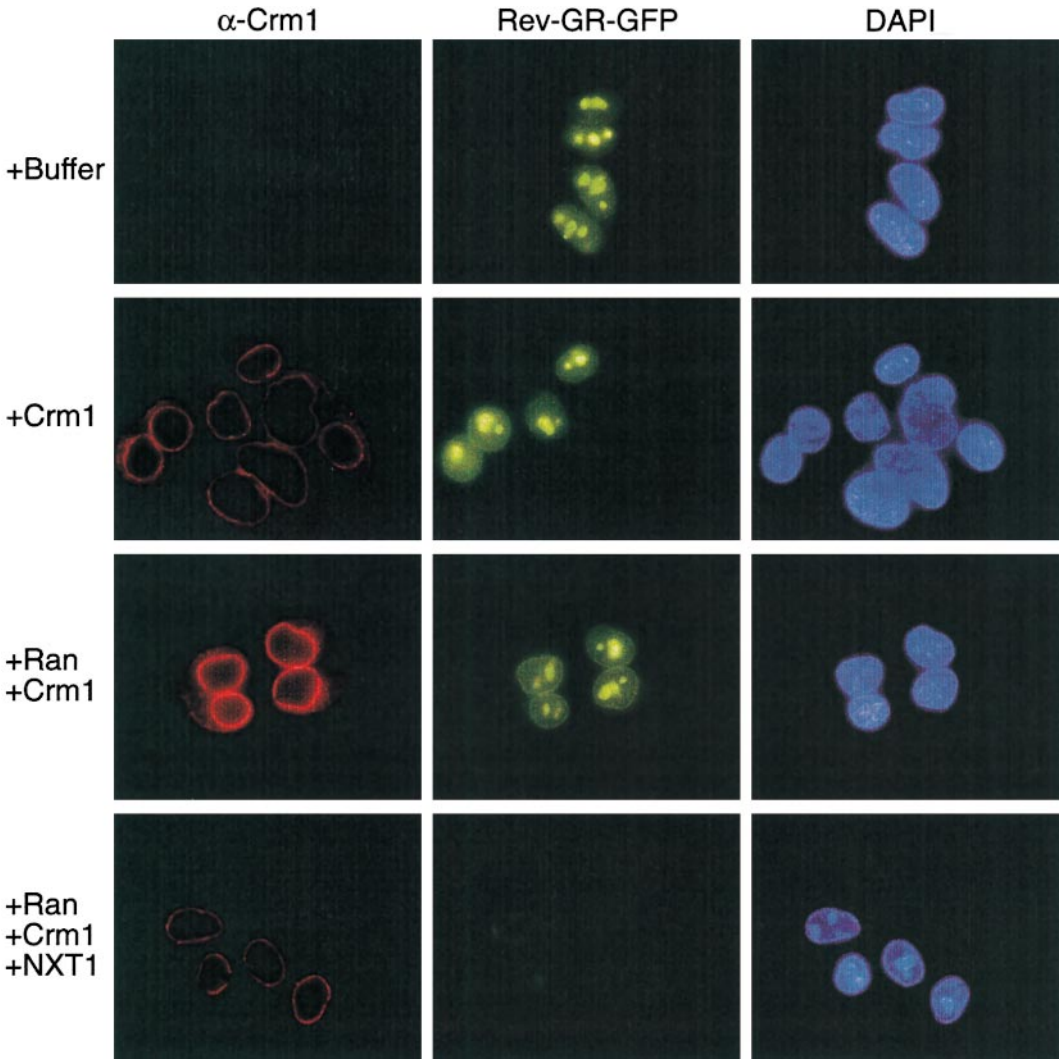


Figure 5. NXT1 stimulates the release of Crm1 from the NPC. Crm1 was detected by indirect immunofluorescence using a Crm1 antibody (Kehlenbach et al., 1998). Because the integrity of the NE was maintained throughout the antibody incubations, the Crm1 signal (red) represents Crm1 that has accumulated on the cytoplasmic face of the NPC. In reactions performed without recombinant transport factors (+Buffer), background levels of Crm1 were detected, probably corresponding to the endogenous export receptor. Crm1 (16 nM) addition results in near background levels of Crm1 detected on the cytoplasmic face of the NPC (+Crm1). The addition of Crm1 (16 nM) and Ran (1.5 μM) to the reaction causes the accumulation of Rev–GR–GFP at the NE. Under these conditions, high levels of Crm1 are visualized on the cytoplasmic face of the NPC (+Ran+Crm1). Including NXT1 (17 μM) in the reaction stimulates the release of Crm1 from the cytoplasmic face of the NPC, and a pronounced increase in total Rev–GR–GFP export (+Ran+Crm1+NXT1). The reduction in Crm1 staining at the NPC probably reflects a NXT1-dependent release reaction, though it is formally possible that NXT1 simply prevents Crm1 accumulation on the cytoplasmic side of the NPC. The latter possibility is unlikely, given that NXT1 addition results in robust stimulation of Rev–GR–GFP export. Export reactions containing recombinant transport factors were supplemented with RanBP1 (1.7 μM).

uble components for this transport pathway. It also implicates NXT1 and RanBP1 as essential factors for the terminal step of nuclear export.

The molecular basis of NXT1-dependent Rev–GR–GFP release from the NPC is likely to reflect its interaction with the export complex containing Ran and Crm1. For example, NXT1 could assemble into the export complex in the nucleoplasm and function as a subunit that specifies targeting to, and release from, a nucleoporin oriented on the cytoplasmic side of the NPC. Alternatively, NXT1 might interact transiently with the export complex after it arrives on the cytoplasmic side of the NPC and promote Rev–GR–GFP release. We addressed this issue in permeabilized cells with a two-step order of addition experiment. In the first step, we added Ran and Crm1 to drive accumulation of Rev–GR–GFP at the antibody accessible site on the cytoplasmic side of the NPC. In the second step, we added the indicated factors, and subsequently determined the level of Rev–GR–GFP on the cytoplasmic side of the NPC. RanBP1 was included in both steps of these reactions, since it promotes nuclear export in our system (Fig. 3). We found that NXT1 addition in the second step had little effect on the level of Rev–GR–GFP detected at the NPC (Fig. 4, Ran+Crm1→NXT1). This suggests that NXT1 does not stimulate Rev–GR–GFP release through a simple transient interaction with the export complex. It also indicates that NXT1 probably interacts with the export complex in an early step of export, before the arrival of the export com-

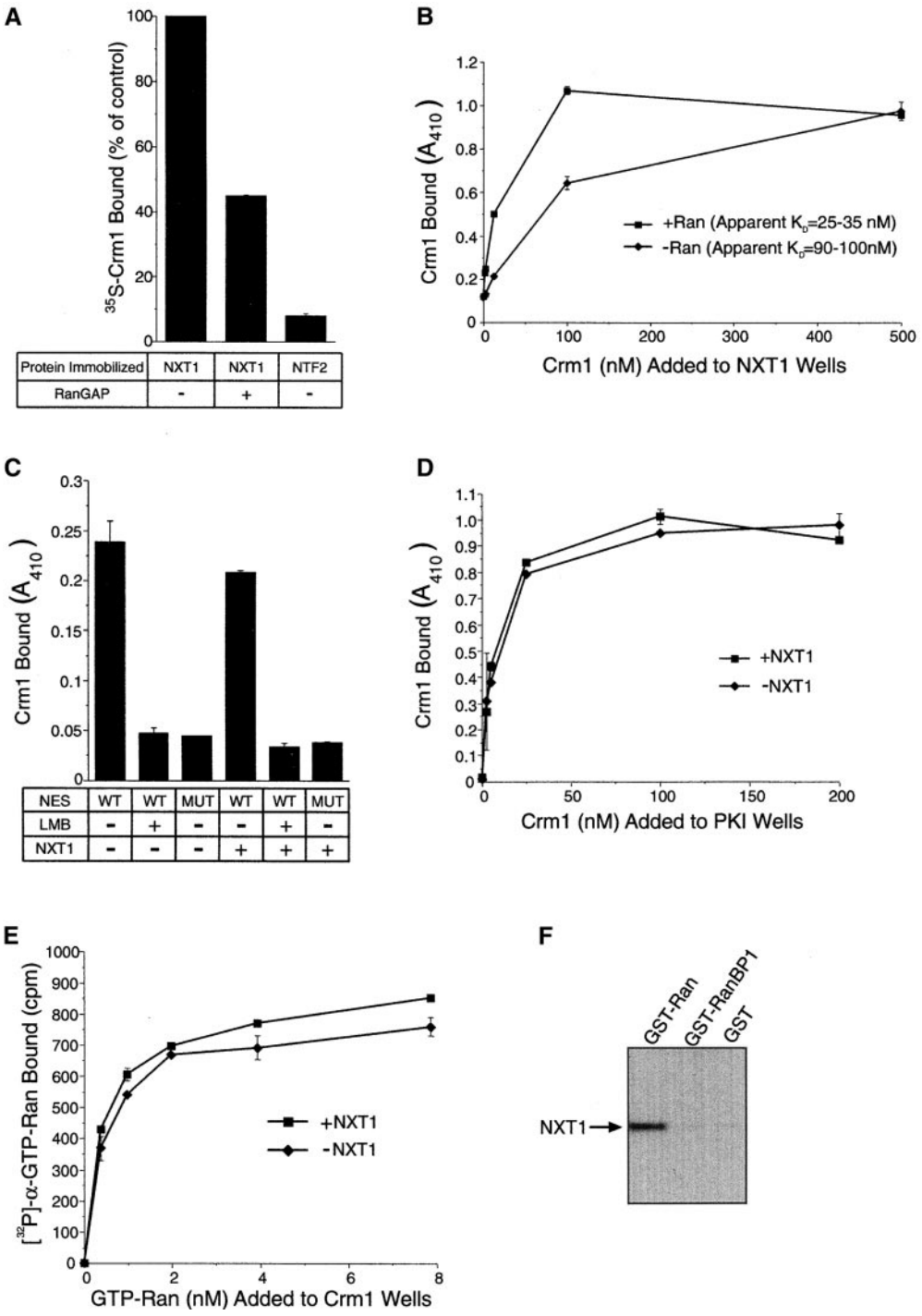


Figure 6. NXT1 binds directly to the export complex. (A) Crm1 specifically interacts with NXT1 in solid-phase binding assays. Values shown correspond to relative amounts of [^{35}S]Crm1 bound as a percent of binding to NXT1 wells in the absence of RanGAP (3,221 ± 215 cpm of [^{35}S]Crm1 bound in this condition). Crm1 binding is 12-fold higher in NXT1 wells compared with the binding to NTF2 wells. Pretreatment of the binding reaction with RanGAP, which converts the Ran-GTP present in the reticulocyte lysate to Ran-GDP, reduces Crm1 binding to NXT1 to 45.5 ± 0.4% of that observed in the absence of RanGAP. (B) NXT1 binds directly to Crm1, and binding is stimulated by the addition of Ran preloaded with GMP-PNP. A concentration range of recombinant Crm1 was added to NXT1 wells in the presence (■) or absence (◆) of 500 nM Ran. A 20-amino acid peptide containing the NES of PKI (500 nM) was included in the binding reactions. (C and D) NXT1 does not affect the affinity of Crm1 for NES substrate. Reactions were performed in the presence of Ran (200 nM) that had been preloaded with GMP-PNP. In C, either wild-type or the NES mutant (L41,44A) (Wen et al., 1995) recombinant PKI protein was immobilized in microtiter wells. Binding reactions containing Crm1 (2.5 nM) that was preincubated in the absence or presence of LMB (500 nM) were added to the PKI wells. The interaction between Crm1 and PKI is specific for the wild-type NES and is blocked by LMB. In D, a concentration range of Crm1 was tested in the presence (■) or absence (◆) of 50 nM NXT1 in PKI-containing wells. (E) NXT1 has little effect on Ran-GTP stabilization on immobilized Crm1. The NES peptide (500 nM) was included in the binding reactions. A concentration range of Ran, preloaded with [α-^{32}P]GTP, was added to wells containing immobilized Crm1 in the presence (■) or absence (◆) of 50 nM NXT1. (F) NXT1 binds to Ran, but not RanBP1. NXT1 (500 nM) was added to wells containing immobilized GST-Ran, GST-RanBP1, or GST. Bound protein was eluted in SDS-PAGE sample buffer, and analyzed by immunobloting using an anti-peptide antibody to NXT1. NXT1 was found to bind directly to GST-Ran, but not to GST-RanBP1 or GST. GST-RanBP1 immobilized in microtiter wells is functional, as assessed by Ran-binding assays (Steggerda and Paschal, 2000).

plex on the cytoplasmic side of the NPC. However, the activity of NXT1 is manifest in a late step of export, which includes substrate release into the cytoplasm.

Because NXT1 is a Ran-GTP–binding protein that shuttles between the nucleus and cytoplasm, we considered the formal possibility that its nuclear export function involves nuclear export of Ran-GTP. That is, NXT1 could facilitate the delivery of Ran-GTP from the nucleoplasm to the cytoplasm. Hypothetically, Ran-GTP could be used to dissociate export complexes on the cytoplasmic side of the

NPC. NTF2 appears to perform what might be considered a reciprocal function, because it facilitates the delivery of Ran-GDP from the cytoplasm to the nucleoplasm, and after nucleotide exchange, Ran-GTP is used to dissociate import complexes on the nucleoplasmic side of the NPC. If the primary function of NXT1 is to deliver Ran-GTP to the cytoplasm, then addition of excess Ran-GTP should promote release of Rev–GR–GFP from the cytoplasmic side of the NPC. However, we found that Ran-GTP addition does not stimulate Rev–GR–GFP release from the NPC (Fig. 4, Ran+Crm1→Ran-GTP). Importantly, the Rev–GR–GFP in this assay is not irreversibly arrested on the cytoplasmic side of the NPC, since it can be released by the addition of cytosol (Fig. 4, Ran+Crm1→Cytosol). This may reflect the presence of a soluble-release activity that acts directly on the export complex. Alternatively, release of Rev–GR–GFP might occur when export complexes assemble from HeLa cytosol–derived Crm1. Newly assembled export complexes could then promote dissociation of preexisting export complexes by competing for a limited number of binding sites on the cytoplasmic side of the NPC. We addressed this using cytosol pretreated with phenyl-Sepharose to remove Crm1 (Holaska and Paschal, 1998). Addition of phenyl-Sepharose–treated cytosol (PS Cytosol) was much less effective than control cytosol in promoting Rev–GR–GFP release (Fig. 4, Ran+Crm1→PS Cytosol), and nucleolar fluorescence remained significantly brighter in the phenyl-Sepharose–treated cytosol samples. Whereas these results do not rule out a role for a soluble release activity, they suggest that cytosolic Crm1 and the ongoing assembly and translocation of export complexes may represent part of the release mechanism for preexisting export complexes.

We addressed whether the function of NXT1 in the terminal step of export also involves the release of the receptor Crm1 from the NPC. We performed Rev–GR–GFP export assays, and monitored the level of Crm1 localized to the cytoplasmic face of the NPC by immunofluorescence microscopy. The antibody approach for Crm1 detection in this experiment is similar to that used to detect Rev–GR–GFP (Figs. 2–4), where the integrity of the NE is maintained. Using a Crm1 antibody (Kehlenbach et al., 1998), we observed a high level of Crm1 accumulation on the cytoplasmic face of the NPC that was dependent upon the addition of recombinant Ran and Crm1 (Fig. 5, +Ran+Crm1). A visible accumulation of Rev–GR–GFP at the NE was only observed in the presence of both Ran and Crm1, suggesting these proteins may be part of an export complex situated on cytoplasmic face of the NPC. Including NXT1 in the reaction caused a substantial reduction in the level of Crm1 on the cytoplasmic face of the NPC (Fig. 5, +Ran+Crm1+NXT1). Therefore, these data indicate that NXT1 stimulation of nuclear export involves release of both the NES receptor (Crm1) and the NES substrate Rev–GR–GFP.

Interactions of NXT1 with the Crm1 Export Complex

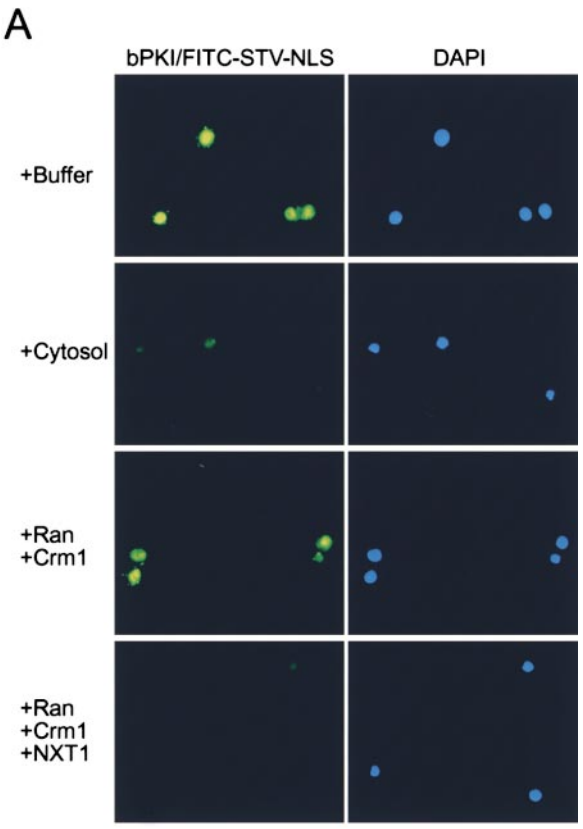
We addressed the hypothesis that NXT1 physically interacts with the export complex in a series of binding experiments. Because NXT1 binds directly to Ran-GTP in vitro (Black et al., 1999), and the latter is a stoichiometric component of the export complex, we considered it possible that Ran-GTP bridges or regulates the interaction between NXT1 and Crm1. We first tested the ability of recombinant NXT1 to bind ^{35}S-labeled Crm1 synthesized in reticulocyte lysate, using NXT1 immobilized in microtiter wells. Crm1 displayed specific binding to NXT1, whereas Crm1 binding to NTF2 was near background (Fig. 6 A). Given that Crm1 and NXT1 are both Ran-GTP–binding proteins, we examined whether the interaction requires the GTP form of Ran. We tested this by pretreating the reaction with excess recombinant RanGAP to convert Ran-GTP to Ran-GDP. This reduced Crm1 binding to 45.5 ± 0.4% of the untreated sample (Fig. 6 A). RanGAP-insensitive binding of Crm1 to NXT1 may represent Ran-GTP–independent interaction between these proteins, or that Crm1-NXT1 complexes have the capacity to protect Ran-GTP from RanGAP action.

We obtained evidence that NXT1 displays both Ran-independent and -dependent interactions with Crm1 by ELISA. Crm1 binding to NXT1 in the absence of Ran is saturable, with an apparent K_D ~95 nM (Fig. 6 B), providing an explanation for the RanGAP-insensitive binding of Crm1 to NXT1 (Fig. 6 A). However, Ran did stimulate Crm1 binding to NXT1, which reduced the apparent K_D of this interaction to ~30 nM (Fig. 6 B). To determine if NXT1 can regulate the interaction of Crm1 with NES substrate, we used an assay to measure direct binding of Crm1 to PKI, performed in the presence of Ran-GTP. Crm1 binding to the immobilized PKI, in this assay, is highly specific, since binding is blocked by LMB and the NES mutant of PKI (L41,44A) shows only background level of binding (Fig. 6 C, MUT). Including NXT1 in the reaction did not affect the level of Crm1 binding to PKI (Fig. 6 C), even when the assay was performed over a 100-fold concentration range of Crm1 (Fig. 6 D). We also examined whether NXT1 affects Ran-GTP binding to Crm1, assayed in the presence of NES substrate. Ran-GTP binding to immobilized Crm1 was saturable and increased only slightly in the presence of NXT1 (Fig. 6 E). Our data demonstrate that whereas NXT1 binds directly to an export complex containing Crm1 and Ran-GTP, it does not to play a primary role in regulating NES recognition or promoting complex assembly.

Since we observed a cooperative effect of NXT1 and RanBP1 in the release of Rev–GR–GFP from the NPC (Fig. 3), we examined if these two proteins can directly interact. We immobilized GST-RanBP1, GST-Ran, and GST in microtiter wells, the latter proteins serving as controls for binding specificity. Recombinant NXT1 bound to GST-Ran, but not to immobilized GST-RanBP1, or to GST. These data suggest the action of NXT1 and RanBP1 in the release of export complexes from the NPC is unlikely to involve a direct interaction between these proteins.

NXT1 Mutants Deficient in Crm1-mediated Export

Our mutational analysis of NXT1 was based on a structural model generated using the crystal structure of NTF2 (Bullock et al., 1996; Black et al., 1999). We designed point mutations (N48E, E102N, and R107A) to alter highly conserved residues in solvent-exposed loops that are predicted to be proximal to the Ran-binding domain of NXT1. We also deleted four amino acids from the COOH terminus of NXT1 (ΔDWAS), since the COOH terminus of NTF2 helps stabilize Ran-GDP binding (Clarkson et al., 1997). Finally, we mutated a phylogenetically conserved lysine (K127A), whose spatial position is predicted to be removed from the Ran-binding surface, in NXT1. We expressed and purified the mutant NXT1 proteins and char-



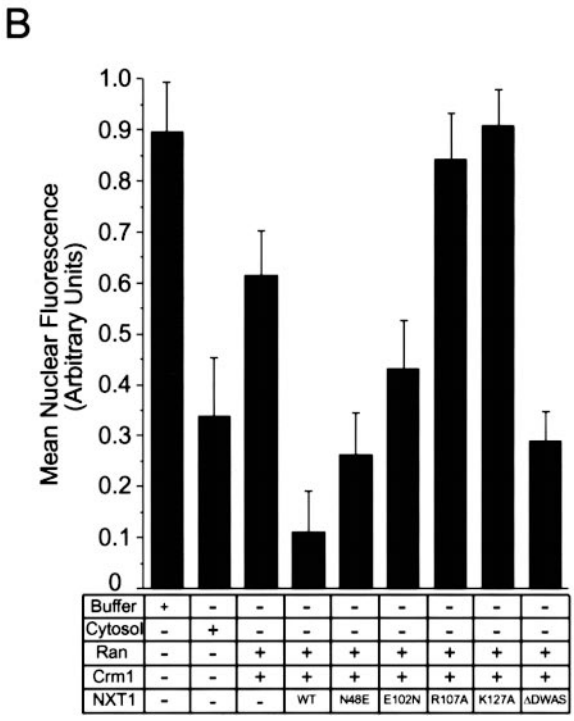


Figure 7. Mutant NXT1 proteins have reduced activity in the PKI export assay. (A) NXT1 stimulates PKI export in the presence of recombinant Ran and Crm1. Fields of cells from PKI export reactions performed with the indicated factors are shown for comparison. (B) Quantitation of NXT1 and NXT1 mutant protein activity in NES-dependent nuclear export. PKI assays were performed, and the level of export was measured in ~50 randomly selected postexport nuclei and expressed as the mean nuclear fluorescence (± SD). Ran was used at 1.3 μM, Crm1 was used at 150 nM, and NXT1 proteins were used at 14 μM.

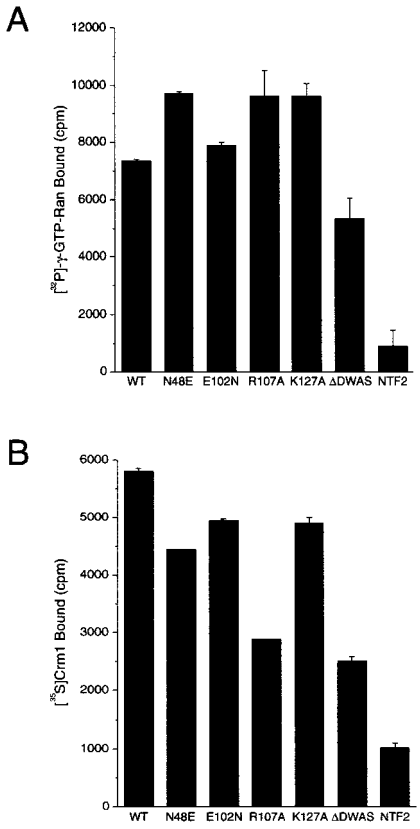


Figure 8. NXT1 mutant proteins deficient in binding Ran and Crm1. (A) Solid-phase Ran-GTP binding assays were performed with immobilized NXT1 mutant proteins. Most NXT1 mutants bind Ran-GTP with near wild-type affinity. However, the COOH-terminal truncation (ΔDWAS) of NXT1 is deficient for Ran-GTP binding. Wild-type NXT1 and NTF2 proteins serve as controls in this experiment. Ran-GTP binding data for the E102N mutant protein has been reported previously (Ossareh-Nazari et al., 2000). (B) The R107A and COOH-terminal truncation NXT1 mutant proteins are deficient in binding Crm1. NXT1 mutant proteins were tested for their ability to bind radiolabeled Crm1 in solid-phase binding assays. The Crm1-binding ability of the R107A mutant protein is reduced, as is that of the COOH-terminal truncation mutant (ΔDWAS).

acterized them using both nuclear export and binding assays, as described below.

We first examined the activity of the mutants using the PKI export assay, which permits quantitative analysis of export activity by measuring nuclear fluorescence at the end of the export reaction (Black et al., 1999). We have described multiple controls to show that the reduction in nuclear fluorescence reflects bona fide nuclear export (Holaska and Paschal, 1998). We have also used the PKI assay previously to show that wild-type NXT1 can stimulate nuclear export of PKI in the presence of a subsaturating concentration of HeLa cytosol (Black et al., 1999). We found that the addition of recombinant Ran, Crm1, and NXT1 is sufficient to promote nuclear export of biotinylated PKI complexed with fluorescent STV (Fig. 7, A and B, bPKI/FITC-STV-NLS). We assayed each of the NXT1 mutant proteins in a similar manner, and determined that the R107A and K127A mutants were severely deficient in their ability to promote nuclear export of the PKI substrate (Fig. 7 B). The E102N mutant was also significantly reduced in export activity, whereas the N48E and ΔDWAS mutants showed an intermediate level of export activity.

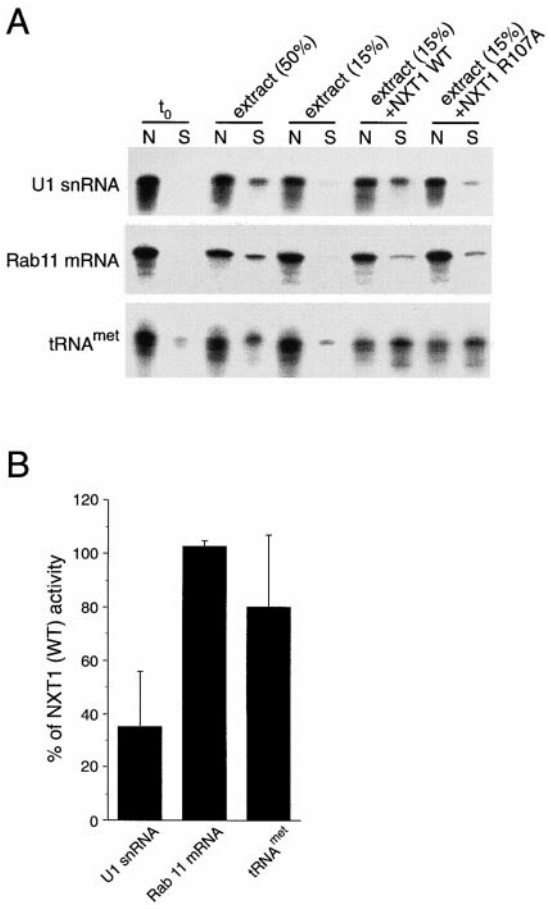


Figure 9. The R107A NXT1 mutant protein promotes mRNA and tRNA export, but is reduced for U1 snRNA export. (A) RNA export assays were performed in permeabilized HeLa cells in the presence of the indicated concentrations of *Xenopus* egg extracts. NXT1 proteins (17 μM) were added to the indicated samples. Samples were separated into nuclear (N) or supernatant (S) fractions and analyzed by denaturing gel electrophoresis. (B) Quantitation of R107A mutant activity in stimulating the export of different classes of RNA. The activity of wild-type (WT) and R107A NXT1 proteins were quantitated by measuring the ratio of RNA released to the supernatant (S) to the total RNA (N+S) and subtracting the export observed in samples that had 15% extracts only. The export activity of the R107A mutant protein is expressed as a percent relative to WT NXT1 activity. Whereas the R107A mutant is capable of stimulating the Crm1-independent export pathways (mRNA and tRNA) at levels similar to WT NXT1, it is deficient in Crm1-dependent U1 snRNA export.

The reduced activity of NXT1 mutants is predicted to be linked to reduced interactions with the export complex containing Ran and Crm1. We evaluated this hypothesis in solid-phase binding assays using wild-type NXT1 and mutant NXT1 proteins immobilized in microtiter wells, and NTF2 as a negative control. With the exception of the COOH-terminal truncation mutant protein ΔDWAS, each of the mutant proteins were able to bind Ran-GTP at levels that were comparable to wild-type NXT1 (Fig. 8 A). We next tested whether these immobilized NXT1 proteins could interact with [^{35}S]Crm1, which was synthesized in reticulocyte lysate. The N48E, E102N, and K127A mutants showed a slight reduction in Crm1 binding. Interestingly, the ΔDWAS mutant that showed a reduction in Ran-GTP binding was deficient for Crm1 binding as well (Fig. 8 B). In contrast, we found that the R107A mutant, which showed a wild-type activity for Ran-GTP binding, was deficient for Crm1 binding. Taken together, our data show that the COOH terminus of NXT1 is important for its export function in permeabilized cells and for its interactions with two components of the export complex, Ran-GTP and Crm1. Arginine 107, which is conserved among all known NXT1 orthologs (Black et al., 1999) and probably located on an exposed loop of the protein, appears to be critical for binding Crm1.

The R107A Mutation in NXT1 Reduces Crm1-dependent RNA Export

We have shown previously that in addition to its role in protein export, NXT1 also functions in RNA export (Ossareh-Nazari et al., 2000). Recombinant NXT1, when added to a permeabilized cell assay that measures nuclear transport of radiolabeled RNA, stimulates export of U1 snRNA, tRNA, and mRNA (Ossareh-Nazari et al., 2000). Based on our present data, the function of NXT1 on these export pathways is predicted to involve its interactions with the export receptors. U1 snRNA export relies on Crm1 as the receptor (Fornerod et al., 1997), explaining why NXT1 stimulates export of this RNA substrate in vitro (Ossareh-Nazari et al., 2000). tRNA export is mediated by the importin-β superfamily member exportin-t, whereas the identification of the receptor(s) for mRNA export remains an area of active investigation. NXT1 has also been shown to be an important cofactor for nuclear export of viral RNA mediated by the receptor TAP (Katahira et al., 1999; Bachi et al., 2000; Kang et al., 2000). Therefore, NXT1 appears to play important roles in both protein and RNA export pathways that involve several different receptors (Ossareh-Nazari et al., 2000).

We examined the effect of the R107A mutation on the export of three different RNA substrates: U1 snRNA, mRNA, and tRNA. NXT1-dependent export of all three RNA substrates is observed when the wild-type protein is assayed in reactions that contain a subsaturating concentration (15% by volume) of *Xenopus* cytosol. The R107A NXT1 mutant showed a threefold reduction in its ability to promote U1 snRNA export (Fig. 9). In contrast, the R107A mutant promoted nuclear export of mRNA to the same level as wild-type NXT1, and the R107A mutant protein was also active in promoting export of tRNA. These results, together with the reduced activity of the R107A mutant in the PKI export assay (Fig. 7) and the Crm1-binding assay (Fig. 8 B), indicate that the export function of NXT1 is specifically linked to its interaction with Crm1. The reduced activity of the NXT1 mutant R107A in the U1 snRNA export pathway, compared with mRNA and tRNA export pathways, suggests it is possible to uncouple its interactions with different export receptors.

Discussion

Here, we have shown that NXT1 is a cofactor for Crm1-mediated protein export from the nucleus. By using recombinant proteins to characterize the factors required for

nuclear export in permeabilized cells, we determined that Crm1 and Ran are sufficient to reconstitute translocation of the NES reporter Rev–GR–GFP from the nucleoli to the cytoplasmic side of the NPC. Addition of NXT1 to the export reaction, containing Crm1 and Ran, results in a significant reduction in the level of Rev–GR–GFP at two topologically distinct sites: within the nucleoli and associated with the cytoplasmic side of the NPC. Thus, NXT1 activity on the Crm1-dependent export pathway is manifest in both the nucleoplasm and in the cytoplasm. The latter clearly reflects NXT1-dependent release of Rev–GR–GFP in a terminal step of export. The NXT1-dependent reduction in nucleolar fluorescence could indicate a role for NXT1 in export complex assembly or targeting to the NPC. Alternatively, it is possible that complex assembly and targeting are delayed until the terminal step of NES-substrate release is completed. NXT1 stimulates nuclear export of diverse macromolecules transported via the Crm1 pathway, which include the Rev and PKI proteins (this study), as well as U1 snRNA (Ossareh-Nazari et al., 2000). It seems likely that NXT1 serves as a cofactor for all NES-containing substrates that undergo Crm1-dependent export from the nucleus.

In addition to its role in Crm1-mediated export, NXT1 also participates directly in viral RNA export mediated by a protein termed TAP (Katahira et al., 1999). Transcripts encoded by the Mason-Pfizer monkey virus contain a CTE that functions as a cis-acting export signal (Hammarskjöld, 1997) that is recognized by TAP in the nucleus (Gruter et al., 1998). A complex of TAP and NXT1 appears to bind CTE-containing RNA and facilitates its export to the cytoplasm. Whether NXT1 performs analogous functions in the Crm1 and TAP export pathways, the latter predicted to involve release of CTE-containing RNA from the cytoplasmic side of the NPC, is an important question for future study.

NXT1 Is a Cofactor for Crm1-dependent Export

The mechanism of NXT1-dependent export is likely to involve its direct interactions with Ran-GTP and Crm1. All three factors are necessary for efficient nuclear export, and NXT1 can bind directly to Ran-GTP (Black et al., 1999) and Crm1 (this study) in vitro. Moreover, the apparent K_D of Crm1 for NXT1 (90–100 nM) is reduced three- to four-fold in the presence of Ran-GTP (25–35 nM), indicating that these proteins display cooperative binding. Independent evidence that Ran-GTP can regulate the interaction between Crm1 and NXT1 was obtained in a solid-phase binding assay using Crm1 translated in reticulocyte lysate. We found that treating the lysate with excess RanGAP to convert Ran-GTP to Ran-GDP significantly reduced Crm1 binding to NXT1. These data imply that Ran-GTP bridges the interaction between Crm1 and NXT1, or that Ran-GTP binding alters Crm1 to a conformation that is more favorable for binding NXT1. Our identification of a mutant NXT1 (R107A) that is wild-type for Ran-GTP binding, but deficient for Crm1 binding, suggests that NXT1 has binding sites for both Ran-GTP and Crm1. We also found that Crm1 binding to PKI in the presence of Ran-GMP-PNP, which in our solid phase assay is specific for a wild-type NES and inhibited by LMB, was unaffected by NXT1. We conclude from this result that NXT1 does not stimulate the initial formation of the export complex, however, we cannot rule out the possibility that NXT1 affects export complex stability, or targeting to the nuclear side of the NPC in vivo.

NXT1 Promotes NES Substrate Release from the NPC

Our experiments clearly demonstrate that NXT1 facilitates the release of NES substrate in the terminal step of nuclear export, which appears to be a rate-limiting step in this and probably all export pathways. The first indication that NXT1 might function in this capacity came from our observation that NXT1 reduces the Crm1/Ran-dependent accumulation of Rev–GR–GFP at the NPC. Because this occurs under conditions where the level of Rev–GR–GFP at nucleoli is also reduced, NXT1 does not act as an inhibitor of Ran/Crm1-dependent delivery of Rev–GR–GFP to the NPC. Rather, NXT1 is a positively acting factor for nuclear export.

We used antibody detection of Rev–GR–GFP to demonstrate that Crm1 and Ran are sufficient for delivery of NES substrate to the cytoplasmic side of the NPC. In the absence of the components necessary to release Rev–GR–GFP from the NPC, we observe an accumulation of the NES substrate on the cytoplasmic side of the NPC. We found that addition of recombinant NXT1 was sufficient to promote a substantial level of Rev–GR–GFP release from the NPC, as assayed by fluorescence microscopy. However, quantitative release of Rev–GR–GFP was only observed in reactions containing HeLa cytosol, or in reactions containing Crm1, Ran, NXT1, and RanBP1 (Fig. 3). This result is interesting in light of the recent proposal that RanBP1 plays a role in the terminal step of nuclear export (Askjaer et al., 1999; Kehlenbach et al., 1999). Ran-GTP in the context of an export complex is protected from RanGAP-stimulated hydrolysis, and the addition of RanBP1 overcomes this protection (Askjaer et al., 1999; Paraskeva et al., 1999), thereby acting as a RanGAP cofactor. Based on these properties and the localization of RanGAP on the cytoplasmic side of the NPC (Matunis et al., 1996; Mahajan et al., 1997), conversion of Ran-GTP to Ran-GDP by RanGAP and RanBP1 could trigger the disassembly of the export complex in the terminal step of nuclear export. Using purified proteins, it has been shown that Crm1 prebound to an immobilized NES in the presence of Ran-GTP can be released only if RanGAP and RanBP1 are added (Askjaer et al., 1999). It has also been found that Crm1, arrested at the NPC in the presence of the Ran mutant Q69L, can be dissociated by addition of RanBP1 or a Ran-binding domain from RanBP2 (Kehlenbach et al., 1999), though this probably reflects a displacement rather than a hydrolysis reaction. Our assay system, which detects the release of Rev–GR–GFP from the cytoplasmic side of the intact NPC, displays a dependence on both NXT1 and RanBP1. NXT1 does not function as a coactivator of RanGAP in vitro (Black et al., 1999), which, together with our current findings, indicates the terminal step of export involves more than Ran-GTP hydrolysis stimulated by RanGAP. Including NXT1 in reactions with Crm1, Ran, and RanBP1 also led to the reduction in the level of Crm1 detected on the cytoplasmic face of the NPC (Fig. 5), indicating the entire export complex is released from the NPC. On this note, it will be of interest to determine how release of the export substrate may be coordinated with the release of Crm1 from the NPC, reactions that could occur either simultaneously or sequentially.

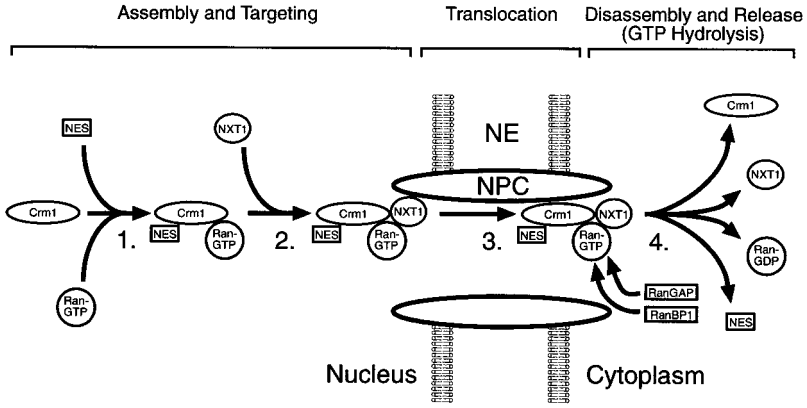


Figure 10. Model for steps in the Crm1-mediated nuclear export pathway. The formation of a ternary complex comprised of Crm1, Ran-GTP, and NES-containing substrate (step 1) occurs in the nucleoplasm, possibly independent of NXT1. NXT1 associates with the complex via direct interactions with Ran-GTP and Crm1, and the complex is delivered to the NPC (step 2). Translocation through the central gated channel of the NPC (step 3) can occur independently of NXT1. However, NXT1 is required for efficient release of the complex from the cytoplasmic side of the NPC (step 4). The export reaction is terminated upon dissociation of the complex; this appears to be facilitated by RanBP1 and RanGAP, or by a concerted action of the two proteins. Conversion of Ran-GTP to Ran-GDP makes the terminal step of export irreversible.

Model of NXT1 Function in NES Export

We have created a working model of nuclear export that integrates our molecular characterization of NXT1 with previous studies on Crm1 and other components of the pathway (Fig. 10). Nuclear export is predicted to initiate in the nucleoplasm with a series of protein–protein interactions that ultimately terminate in the cytoplasm with the release of NES substrate. The first step is Crm1 binding to NES substrate, which is stabilized by Ran-GTP. We propose that a second step of export includes the assembly of NXT1 into the complex in the nucleoplasm, which is followed by its targeting to the NPC. The third step of export is translocation of the complex through the central channel of the NPC. The fourth step is the disassembly of the complex, which results in NES substrate release into the cytoplasm.

Our data supports the notion that NXT1 is not required for the earliest step in the pathway, that is, the binding of NES substrate to Crm1 in a complex that includes Ran-GTP. This is based on our observation that NXT1 does not affect Crm1 binding to PKI (Fig. 6 C). However, we propose that NXT1 engages the export complex before its delivery to the cytoplasmic side of the NPC, based on two results. First, in the order of addition experiment, NXT1 stimulates little release of Rev–GR–GFP that is preassembled in a complex with Crm1 and Ran on the cytoplasmic side of the NPC. In contrast, when NXT1, Crm1, and Ran-GTP are added simultaneously to the reaction, we observe nearly complete release of Rev–GR–GFP from the NPC. This suggests that to exert its full release activity, NXT1 needs to interact with the export complex before its arrival on the cytoplasmic side of the NPC. Second, NXT1 addition reduces the level of Rev–GR–GFP at the nucleolus. We believe this indicates that NXT1 promotes a reaction that precedes Rev–GR–GFP release, such as targeting to the nuclear side of the NPC. It is also formally possible that NXT1 reduces the level of Rev–GR–GFP at the nucleolus by an indirect mechanism. For example, export complex assembly in the nucleolus might be impeded until substrate release from the cytoplasmic side of the NPC is completed. This could be coordinated by a limiting component for export complex assembly such as Crm1 that has to be recycled from the cytoplasmic side of the NPC to the nucleolus. Because NXT1 facilitates the release of Crm1 from the NPC, NXT1 could be involved, albeit indirectly, in such a mechanism. However, it is unlikely that the primary function of NXT1 involves reimport of Crm1, since Crm1 can undergo both import and export in our assay system in the absence of NXT1. Nonetheless, linking export complex assembly, substrate release, and recycling is an attractive mechanism for coordinating the distribution and activity of export receptors and their regulators.

The current model of the terminal step of nuclear export, release of NES substrate from the cytoplasmic side of the NPC, relies on Ran-GTP as a key regulatory subunit that dictates the assembly state of the export complex. RanBP1 and related domains in RanBP2 can destabilize, and possibly dissociate, the interaction between Ran-GTP and Crm1 in the export complex. Ran-GTP is essential for high-affinity binding of Crm1 to NES substrate (Fornerod et al., 1997), and RanBP1 addition can stimulate the dissociation of these proteins (Fig. 3) (Kehlenbach et al., 1999). Subsequent RanGAP-stimulated hydrolysis of Ran-GTP to Ran-GDP makes the release reaction irreversible, because Crm1 fails to bind Ran-GDP. Our analysis revealed that RanBP1 was insufficient to stimulate the release reaction, as it had a negligible effect in our assay, unless NXT1 was included as well (Fig. 3). This indicates that NXT1 and RanBP1 perform distinct functions in the release reaction. One possibility is that NXT1 promotes transfer of the export complex from a proximal accumulation site to a more distal site, where RanBP1, RanBP2, and RanGAP can complete the release reaction. In this scenario, NXT1, bound to an export complex, would facilitate its forward progress within the NPC. Determining whether NXT1- and RanBP1-facilitated NES substrate and receptor release occur through a single reaction step or through sequential reaction steps, and mapping the location of these intermolecular events within the substructure of the NPC, should provide significant insight into the mechanism of nuclear export.

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