Interaction Between NTF2 and xFxFG-containing Nucleoporins is Required to Mediate Nuclear Import of RanGDP

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Nuclear transport factor 2 (NTF2) is a small, homodimeric protein that binds to both RanGDP and xFxFG repeat-containing nucleoporins, such as yeast Nsp1p and vertebrate p62. NTF2 is required for efficient nuclear protein import and has been shown to mediate the nuclear import of RanGDP. We have used the crystal structures of rat NTF2 and its complex with RanGDP to design a mutant, W7A-NTF2, in which the affinity for xFxFG-repeat nucleoporins is reduced while wild-type binding to RanGDP is retained. The 2.5 Å resolution crystal structure of W7A-NTF2 is virtually superimposable upon the wild-type protein structure, indicating that the mutation had not introduced a more general conformational change. Therefore, our data suggest that the exposed side-chain of residue 7 is crucial to the interaction between NTF2 and xFxFG repeat-containing nucleoporins. Consistent with its reduced affinity for xFxFG nucleoporins, fluorescently labelled W7A-NTF2 binds less strongly to the nuclear envelope of permeabilized cultured cells than wild-type NTF2 and, when microinjected into *Xenopus* oocytes, colloidal gold coated with W7A-NTF2 binds less strongly to the central channel of nuclear pore complexes than wild-type NTF2-coated gold. Significantly, W7A-NTF2 only weakly stimulated the nuclear import of fluorescein-labelled RanGDP, providing direct evidence that an interaction between NTF2 and xFxFG repeat-containing nucleoporins is required to mediate the nuclear import of RanGDP.

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Introduction

The trafficking of macromolecules, ions and small molecules between nucleoplasm and cytoplasm is mediated by nuclear pore complexes (NPCs), cylindrical proteinaceous structures, about 1200 Å in diameter and 700 Å thick, that perforate the nuclear envelope (reviewed by Pante & Aebi, 1996; Yang *et al.*, 1998). Proteins and nucleic acids are transported actively along the central axis of the NPC (Feldherr *et al.*, 1984; Dworetzky &

Feldherr, 1988) if they contain an appropriate signal. For example, proteins containing a nuclear localization sequence (NLS) are imported into the nucleus, whereas proteins containing a nuclear export sequence (NES) are exported to the cytoplasm. Although there are several different nuclear trafficking pathways, they share a number of common features (reviewed by Görlich, 1998; Mattaj & Engelmeier, 1998; Izaurralde & Adam, 1998; Stewart & Rhodes, 1999). In each case, the substrate or cargo does not interact directly with the NPC, but instead is transported bound to a soluble carrier molecule, which then has to be recycled back to the original compartment. For example, proteins containing a nuclear import signal first attach to soluble receptors of the importin-β family, either directly (Pollard *et al.*, 1996) or *via* an adapter such as importin-α (Görlich *et al.*, 1994). The importin-cargo complex then docks at the

Abbreviations used: NPC, nuclear pore complex; NTF2, nuclear transport factor 2; WGA, wheat germ agglutinin; NLS, nuclear localization sequence; NES, nuclear export sequence; PBS, phosphate-buffered saline; GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein.

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cytoplasmic face of the NPC, before being translocated through the NPC into the nucleus. Translocation is followed by displacement of substrate from the carrier molecule by RanGTP in the nucleus, after which the carrier, complexed with RanGTP, is recycled back through the NPCs to the cytoplasm where RanBP1 and RanGAP act to dissociate the complex and promote hydrolysis of the GTP bound to Ran (Bischoff & Görlich, 1997; Floer & Blobel, 1997; Lounsbury & Macara, 1997). Analogous pathways have been identified for the export of RNA and protein from the nucleus (reviewed by Izaurralde & Adam, 1998; Mattaj & Engelmeier, 1998). Although the association and dissociation of the carrier-cargo complexes have been characterized, the precise molecular mechanism of translocation through NPCs has remained obscure.

The nucleotide state of the Ras-family GTPase Ran is an important determinant of nuclear trafficking (reviewed by Koepp & Silver, 1996; Görlich, 1998). GTP hydrolysis by Ran does not appear to be linked directly to transport (Kose *et al.*, 1997; Nakielny & Dreyfuss, 1998; Ribbeck *et al.*, 1999; Engelmeier *et al.*, 1999; Schwobel *et al.*, 1998) and instead could be used, for example, to specify directionality (see Görlich, 1998) or to sort molecules prior to transport (see Stewart, 1992; Stewart & Rhodes, 1999). The nucleotide state of Ran is determined primarily by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). The principal Ran GEF, RCC1 (Bischoff & Ponstingl, 1991), which recharges the molecule with GTP, is nuclear (Ohtsubo *et al.*, 1989), whereas the principal GAP, RanGAP1 (Bischoff *et al.*, 1994), is cytoplasmic with a proportion attached to fibrils that emanate from the NPCs (Hopper *et al.*, 1990; Melchior *et al.*, 1993; Matunis *et al.*, 1996; Mahajan *et al.*, 1997). This distribution of Ran GEF and GAP has led to the proposal that cytoplasmic Ran is primarily in its GDP-bound state, whereas nuclear Ran is in its GTP-bound form (reviewed by Koepp & Silver, 1996; Görlich, 1998). Moreover, it has been proposed that this Ran nucleotide gradient specifies directionality of transport (Görlich *et al.*, 1996).

Efficient nuclear protein import using the importin- β pathway also requires NTF2 (nuclear transport factor 2, also called p10), a 15 kDa dimeric protein that is essential in yeast and which binds to both RanGDP and to a series of NPC proteins (nucleoporins) that contain distinctive repeating sequence motifs (Moore & Blobel, 1994; Paschal & Gerace, 1995; Corbett & Silver, 1996; Clarkson *et al.*, 1997). These sequence motifs are based on highly conserved cores, containing one or two phenylalanine residues, linked by hydrophilic spacers of very variable sequence but which are rich in charged and polar residues. The two most common cores are based on xFxFG (where x is usually a small polar residue or glycine) and GLFG (reviewed by Rout & Wente, 1994). In vertebrates, many xFxFG repeat nucleoporins bind wheat germ agglutinin (WGA). Nuclei assembled from *Xenopus*

egg extract depleted of WGA-binding proteins do not retain the ability to import NLS-containing substrates, but this ability is restored by adding back material eluted from WGA-sepharose (Finlay & Forbes, 1990), indicating that either the WGA-binding xFxFG nucleoporins, or proteins bound to them, are required for transport. Electron microscopy has indicated that xFxFG nucleoporins are located in the central channel of NPCs and also on both their nucleoplasmic and cytoplasmic faces (Pante *et al.*, 1994; Pante & Aebi, 1996; Grote *et al.*, 1995; Fahrenkrog *et al.*, 1998). Depletion of cytoplasm by the vertebrate xFxFG nucleoporin p62 bound to Sepharose, inhibits nuclear protein import in permeabilized cells (Paschal & Gerace, 1995), but transport can be restored to normal levels by adding exogenous NTF2. Although NTF2 has no detectable affinity for RanGTP (Paschal *et al.*, 1996), it binds RanGDP to form a defined complex (Moore & Blobel, 1994; Paschal & Gerace, 1995; Stewart *et al.*, 1998a). The interaction between NTF2 and RanGDP is crucial for efficient nuclear protein import (Moore & Blobel, 1994; Paschal & Gerace, 1995; Paschal *et al.*, 1996, 1997; Nehrbass & Blobel, 1996; Clarkson *et al.*, 1996, 1997; Corbett & Silver, 1996; Wong *et al.*, 1997). Recent work has indicated that a key role of NTF2 is to mediate nuclear import of RanGDP (Smith *et al.*, 1998; Ribbeck *et al.*, 1998), and so may function in a manner analogous to RabGDI (reviewed by Pfeffer *et al.*, 1995) to direct RanGDP to the nucleus for nucleotide exchange. Efficient nuclear import of Ran is essential for transport because importin β -like transport receptors exit the nucleus complexed with RanGTP and thereby continuously deplete Ran from the nucleus. However, the precise mechanism by which NTF2 facilitates Ran's nuclear import is unknown.

NTF2 binds to RanGDP and xFxFG repeat-containing nucleoporins *in vitro* at different, non-overlapping sites (Moore & Blobel, 1994; Paschal & Gerace, 1995; Paschal *et al.*, 1996; Clarkson *et al.*, 1996, 1997) and mutants of NTF2 or Ran that ablate the NTF2-Ran interaction disrupt the NTF2-mediated import of RanGDP (Smith *et al.*, 1998; Ribbeck *et al.*, 1998). NTF2 exists in solution as a dimer and each chain has an unusual fold based on a bent β -sheet backed by a long α -helix that generates a pronounced hydrophobic pocket (Bullock *et al.*, 1996). The crystal structure of the NTF2-RanGDP complex (Stewart *et al.*, 1998a) indicates that the primary interaction interface is formed by the switch II loop (residues 67-78) of Ran with a smaller contribution from the switch I region (residues 40-43), together with the hydrophobic cavity and surrounding negatively charged residues of NTF2.

Although the precise mechanism by which cargo-carrier complexes are translocated through the NPC has not yet been established, a considerable body of indirect evidence suggests that interactions involving xFxFG repeat-containing nucleoporins may be important. Addition of

bacterially expressed fragments of several xFxFG nucleoporins inhibits nuclear protein import and/or export (Bastos *et al.*, 1996; Iovine *et al.*, 1996; Stutz *et al.*, 1996; Shah & Forbes, 1998) and several xFxFG nucleoporins, including Nup1p, Nup2p, Nup98 and Nup153, bind importin- β family members *in vitro* (Radu *et al.*, 1995a,b; Rexach & Blobel, 1995; Moroianu *et al.*, 1995; Shah & Forbes, 1998; Shah *et al.*, 1998). However, it is not clear that the effects observed with added bacterially expressed nucleoporin repeat constructs are generated directly by an interaction between them and the carrier-cargo complex, as these constructs could either bind to and so obstruct other NPC components, or sequester other soluble transport factors. Therefore, although there have been indications that the nucleoporin repeats may be sites of interaction between carrier molecules and NPCs, direct evidence for their involvement in nucleocytoplasmic transport has been lacking.

Here we describe the W7A mutant of NTF2 in which the interaction with xFxFG nucleoporins is greatly reduced while its ability to bind RanGDP is retained. X-ray crystallography shows that this mutation introduced a negligible structural change, indicating that Trp7, which is exposed on the surface (Bullock *et al.*, 1996), is close to the binding site of the xFxFG repeat cores. W7A-NTF2 binds more weakly to NPCs than wild-type NTF2, especially in the central channel, and mediates the nuclear import of RanGDP less efficiently, providing direct evidence for the involvement of the NTF2-nucleoporin interaction in this transport process. Consistent with this evidence, a soluble construct of yeast nucleoporin Nsp1p containing 19 xFxFG repeats is able to reduce substantially the binding of NTF2 to the nuclear envelope of permeabilized cells and inhibit the nuclear import of Ran.

Results

Expression and characterization of W7A-NTF2

To evaluate the role of the interaction between NTF2 and xFxFG nucleoporins in RanGDP nuclear import, we generated a NTF2 mutant that reduced the interaction with nucleoporins while preserving

Ran binding. Previous work has shown that RanGDP and xFxFG-repeat nucleoporins bind to separate non-overlapping sites on NTF2 (Clarkson *et al.*, 1996). Moreover, because many different xFxFG-repeat nucleoporins bind to NTF2, it appears that the interaction involves primarily the xFxFG core of the repeats rather than the intervening hydrophilic linker regions that vary considerably in sequence (Clarkson *et al.*, 1997). Inspection of the crystal structures of rat NTF2 (Bullock *et al.*, 1996) and a series of mutants (Clarkson *et al.*, 1997), together with the structure of the NTF2-RanGDP complex (Stewart *et al.*, 1998a) indicated that there was a hydrophobic patch on the surface of the NTF2 molecule, centred roughly on Trp7, which remained exposed in the NTF2-RanGDP complex and so was a candidate for binding to the hydrophobic aromatic side-chains of the xFxFG-repeat cores. To investigate this hypothesis, we mutated Trp7 to Ala and examined its influence on the interactions with both RanGDP and xFxFG nucleoporins.

Bacterially expressed W7A-NTF2 eluted from a S100 gel-filtration column at the same volume as wild-type NTF2, corresponding to a M_r of 31 kDa, showing that, like the wild-type protein (Paschal & Gerace, 1995; Bullock *et al.*, 1996), W7A-NTF2 was dimeric in solution. The ability of W7A-NTF2 to dimerize indicated that its conformation was probably similar to that of the wild-type protein. To confirm that the mutation had not introduced any major conformational change into the molecule, we solved its structure to 2.5 Å resolution using X-ray crystallography. The mutant protein crystallized in space group $P2_1$ (Table 1) whereas the native protein crystallized in space group $P2_12_12_1$. The crystals of W7A-NTF2 diffracted to 2.5 Å using synchrotron radiation and produced a high quality data set (Table 1) that was used to generate a molecular replacement solution using the co-ordinates of wild-type NTF2 (Bullock *et al.*, 1996). Refinement using REFMAC (CCP4, 1994) produced a model having a final R -factor of 20.8% (R_{free} 24.0%) and good stereochemistry (Table 2). Figure 1(a) shows a portion of the $2F_o - F_c$ electron density map which was unambiguous in the region of residue 7. The 2.5 Å structure of W7A-NTF2 showed no significant changes from wild-type apart from the

Table 1. Crystallographic data for W7A-NTF2

Lattice constants (Å, deg.)	$a = 64.65$ $\alpha = 90.00$	$b = 75.41$ $\beta = 115.00$	$c = 64.54$ $\gamma = 90.00$
Symmetry	$P2_1$		
Resolution range (Å) ^a	22.7–2.5 (2.64–2.5)		
Number of observations	65,817		
Number of unique reflections	18,928		
Completeness (%) ^a	97.4 (91.8)		
Multiplicity ^a	2.5 (2.3)		
R_{merge} (%) ^{a,b}	8.2 (19.4)		
I/σ ^a	6.1 (3.5)		

^a The highest resolution shell is in parenthesis.

^b $R_{\text{merge}} = \sum_{hkl} \sum_i |I_{hkl} - \langle I_{hkl} \rangle| / \sum_{hkl} \sum_i \langle I_{hkl} \rangle$ where $\langle I_{hkl} \rangle$ is the mean of the observations $I_{hkl,i}$ of reflection hkl .

Figure 1. (a) The $2F_o - F_c$ electron density map of the area in the vicinity of residue 7. (b) Graph of the C α rms difference between the A-chains of wild-type and W7A-NTF2. The structures are virtually superimposable except for the extreme C terminus which previous work (Bullock *et al.*, 1996; Stewart *et al.*, 1998a) has shown to have a variable conformation.

mutated side-chain (Figure 1(b)) and at the C terminus after residue 122. The C terminus of NTF2 is thought to be flexible and differs in conformation between both chains in wild-type NTF2 (Bullock *et al.*, 1996) and in NTF2-RanGDP complex crystals (Stewart *et al.*, 1998a). Pairwise comparisons for C α atom rms deviation for residues 5-120 between the two chains in the wild-type NTF2 asymmetric unit and the four chains in the W7A-NTF2 asymmetric unit ranged between 0.37 and 0.48 Å. Therefore, because the mutation did not introduce an extensive conformational change, the crystal structure indicates that the effects observed with W7A-NTF2 were due directly to the altered side-chain.

The W7A mutation reduces binding of NTF2 to xFxFG repeat nucleoporins while retaining binding to RanGDP

We used both fluorescence and Sepharose-bead binding assays (Clarkson *et al.*, 1996, 1997; Wong *et al.*, 1997) to test the effect of the W7A mutation on the molecular interactions of NTF2. Both wild-type and W7A-NTF2 protein coupled to Sepharose

beads bound RanGDP to comparable levels (Figure 2(a)), whereas constructs corresponding to 19 xFxFG repeats of yeast nucleoporin Nsp1p (Clarkson *et al.*, 1996) or 11 xFxFG repeats of murine nucleoporin p62 (Buss & Stewart, 1995) bound strongly to wild-type NTF2-Sepharose beads but bound very weakly to W7A-NTF2 beads (Figure 2(a)). Similarly, whereas wild-type NTF2 bound to the Nsp1p xFxFG repeat-containing construct coupled to Sepharose, W7A-NTF2 bound much less strongly (Figure 2(b)). In summary, the solution binding assays gave a clear indication that although W7A-NTF2 bound RanGDP in a similar manner to the wild-type protein, the mutant's binding to the xFxFG repeats of Nsp1p and p62 was substantially reduced.

We used fluorescence quenching and bead-binding assays to obtain a more quantitative picture of the effect of the W7A mutation on the interaction between NTF2 and xFxFG repeats (Figure 3). Because NTF2 is a dimer and the xFxFG repeat-containing constructs contained a number of repeats, strictly one can only measure an avidity (analogous to that frequently employed in, for

Table 2. Refinement data for W7A-NTF2 crystals

Resolution range (Å)	6-2.5
Number of residues	494
Number of water molecules	84
R-factor (%) ^a	20.8
Free R-factor (%) ^b	24.0
Bond length rms (Å)	0.012
Bond angle rms (deg.)	2.0
Ramachandran plot	
Most favoured (%)	88.7
Allowed (%)	9.5
Generously allowed (%)	1.4
Forbidden (%) ^c	0.5

^a R-factor = $100 \sum_{hkl} [|F_o(hkl)| - |F_c(hkl)|] / \sum_{hkl} |F_o(hkl)|$ where F_o and F_c are the observed and calculated structure factors, respectively.

^b Free R-factor was computed using 5% of the data assigned randomly.

^c Asp92 was also in the forbidden region in wild-type NTF2 structure (Bullock *et al.*, 1996) as a result of its being in a very tight loop.

example, antibody-antigen interactions) and thus obtain an operational K_d for each interaction. However, although such an operational K_d cannot be interpreted precisely in an absolute sense, it is a very effective measure of the relative strength of the interaction when comparing wild-type and

mutant protein. Moreover, these assay conditions closely parallel the conditions likely to be found in NPCs where many of the nucleoporins that line the central channel contain multiple xFxFG repeats. For wild-type NTF2, bead-binding assays gave similar operational K_d values (of the order of 2-3 μ M) for both p62 and Nsp1p constructs and comparable values were obtained for the Nsp1p-NTF2 interaction using fluorescence assays (the poor solubility and fluorescence properties of the p62 xFxFG constructs frustrated measuring its K_d accurately by fluorescence). Because we saw no evidence for co-operativity in this interaction, we assumed, as a first approximation, that the xFxFG-binding site on each chain of the NTF2 dimer behaved independently. On this basis, the fluorescence data for the Nsp1p construct binding to NTF2 were consistent with a K_d of $1.9(\pm 0.2)$ μ M ($\pm$ standard deviation) and the bead-binding data with a K_d of $3.2(\pm 1.0)$ μ M, whereas bead binding for the p62 xFxFG construct gave a K_d of $1.4(\pm 0.3)$ μ M. If indeed there was a level of co-operativity in the interaction, possibly arising from the multidentate nature of the nucleoporin ligand, the true K_d would be even weaker, and so the K_d

Figure 2. Interaction of W7A-NTF2 with nucleoporin constructs containing xFxFG repeats and RanGDP in solution binding assays using Coomassie-stained SDS-PAGE of protein bound to sepharose beads. (a) Nsp1p (Clarkson *et al.*, 1996) binds to W7A-NTF2 (lane 2) much less strongly than wild-type NTF2 (lane 3), whereas RanGDP binds equally to W7A-NTF2 (lane 4) and wild-type protein (lane 5). A mixture of RanGDP and Nsp1p bind simultaneously to wild-type NTF2 (lane 7), whereas from this mixture only RanGDP shows significant affinity for W7A-NTF2 beads (lane 6). As negative controls, neither the 19 Nsp1p xFxFG repeats (lane 8) nor RanGDP (lane 9) bind significantly to ovalbumin-Sepharose beads. (b) Similarly, the xFxFG repeat-containing N terminus of p62 (Buss & Stewart, 1995) also binds W7A-NTF2 much more weakly than wild-type. (c) Whereas wild-type NTF2 (lane 1) binds to Nsp1p-Sepharose beads, W7A-NTF2 (lane 2) shows no detectable binding. Note that, although NTF2 molecules were covalently coupled to the Sepharose beads, because it is a dimer, one of the subunits dissociated into the protein sample buffer and is visible by SDS-PAGE. Ovalbumin and Nsp1p are monomeric and therefore when coupled to Sepharose beads are not visible by SDS-PAGE (Clarkson *et al.*, 1996).

Figure 3. Measurements of binding interaction between W7A and wild-type NTF2 with a construct of

values quoted should be taken as upper limits for the strength of this interaction.

As shown in Figure 3, W7A-NTF2 bound to the Nsp1p or p62 xFxFG constructs much more weakly than wild-type in either assay system. The W7A binding was so weak that it could not be saturated, and may well have been non-specific. It was not possible to determine binding constants precisely (Figure 3), but modelling the W7A data as the first part of a binding isotherm indicated that the minimum value for the K_d for Nsp1p binding was of the order of 80 μM (fluorescence assays) or 45 μM (bead-binding assays). Because it was likely that the mutated tryptophan residue was making a major contribution to the fluorescence signal, the operational K_d obtained for W7A-NTF2 using this method may not have been estimated accurately, and so it was reassuring that the bead-binding assays also showed a substantially reduced binding of the mutant to xFxFG repeats compared with wild-type NTF2. Consistent with the observation that binding to Nsp1p was at least an order of magnitude weaker for W7A-NTF2, a minimum value for the K_d of p62 binding to W7A-NTF2 of 10 μM (bead-binding assay) was estimated, again an order of magnitude weaker than that measured for the wild-type protein.

W7A-NTF2 binds less strongly to the nuclear envelopes of permeabilized cells

Previous work using light and electron microscopy has shown that NTF2 stains nuclear envelopes in a punctate pattern consistent with its binding to NPCs (Corbett & Silver, 1996; Feldherr *et al.*, 1998; Ribbeck *et al.*, 1998). To evaluate the effect of the W7A mutation on nuclear envelope binding, we labelled wild-type and W7A-NTF2

yeast nucleoporin Nsp1p containing 19 xFxFG-repeats (Clarkson *et al.*, 1996) by increase in intrinsic tryptophan fluorescence of (a) NTF2 and (b) Nsp1p-coupled Sepharose bead-binding assay. (a) The increase in tryptophan fluorescence of wild-type NTF2 is saturable and much greater than for W7A-NTF2. Modelling the W7A data as the first part of a binding isotherm indicated that the minimum value for the K_d was at least two orders of magnitude weaker than the K_d determined for wild-type NTF2. (b) Similarly, wild-type NTF2 binding to Nsp1p-beads was saturable and produced a value for the K_d similar to that derived from the fluorescence assay. Again, W7A-NTF2 binding was not saturable, and modelling the data as before gave a minimum value for the K_d at least an order of magnitude weaker than the K_d determined for wild-type NTF2. (c) Whereas binding of the p62 construct containing FxFG-repeats to NTF2-sepharose beads was saturable, producing a value of the K_d of the same order of magnitude as that for the interaction between NTF2 and the Nsp1p construct, binding of the p62 construct to W7A-NTF2 sepharose beads was not saturable and was approximately an order of magnitude weaker than wild-type.

with fluorescein (Ribbeck *et al.*, 1998), which did not alter the binding to either RanGDP (Ribbeck *et al.*, 1998) or xFxFG repeats (data not shown) in bead-binding assays. When added to digitonin-permeabilized HeLa cells, the nuclear envelope staining of fluorescein-labelled W7A-NTF2 was reduced dramatically compared to wild-type NTF2 (Figure 4). Moreover, previous work (Ribbeck *et al.*, 1998) showed that the amount of fluorescent NTF2 bound to the nuclear envelope is enhanced by the addition of RanGDP and indeed both wild-type NTF2 and the W7A mutant showed such an enhancement, although here too the W7A-NTF2 staining was still much less intense than that of the wild-type (Figure 4). Thus, these fluorescence microscopy data indicate that W7A-NTF2 binds to nuclear envelopes less strongly than the wild-type protein.

W7A-NTF2 shows reduced binding to the central channel of NPCs

We also used electron microscopy to investigate the differences in nuclear envelope binding between wild-type and W7A-NTF2. Previous work (Feldherr *et al.*, 1998) has shown that when colloidal gold particles coated with NTF2 are microinjected into *Xenopus* oocytes, the particles bind to

NPCs and show accumulation in the central channel through which material is transported (Feldherr *et al.*, 1984; Dworetzky & Feldherr, 1988) as well as at the cytoplasmic and nucleoplasmic faces. As shown in Figure 5 and Table 3, with W7A-NTF2, although there was still binding to the nucleoplasmic and cytoplasmic faces, the proportion of protein-coated colloidal gold bound to the central channel of the NPCs was reduced substantially (38 %) compared with wild-type NTF2. Reduced binding to the central channel of the NPCs would also be consistent with the reduced strength of the NTF2-nucleoporin interaction in the W7A mutant (Figures 2 and 3), because the xFxFG repeat-containing nucleoporins to which NTF2 binds (but to which W7A-NTF2 does not) are known to be primarily located along the central channel with some also located at their nucleoplasmic and cytoplasmic faces (Grote *et al.*, 1995; Fahrenkrog *et al.*, 1998; Pante & Aebi, 1996).

W7A-NTF2 mediates RanGDP import less efficiently than wild-type

Previous studies have shown that NTF2 mediates the nuclear import of RanGDP (Smith *et al.*, 1998; Ribbeck *et al.*, 1998) and that the NTF2-RanGDP interaction is essential for efficient nuclear protein import (Clarkson *et al.*, 1997). We therefore examined the effect of the W7A mutation, with its consequent reduction in the strength of the NTF2-xFxFG repeat interaction, on RanGDP nuclear import. The import of fluorescein-labelled Ran (Ribbeck *et al.*, 1998) was assayed in the absence of NTF2, in the presence of wild-type NTF2, and with W7A-NTF2 (Figure 6). In the absence of NTF2, the fluorescent RanGDP stained the nuclear envelope weakly, but was not imported into the nucleus, whereas in the presence of 1.5 μ M wild-type NTF2, fluorescent RanGDP was imported efficiently. By contrast, 1.5 μ M W7A-NTF2 mediated RanGDP import much less efficiently than 1.5 μ M wild-type NTF2 and showed only marginally higher nuclear Ran accumulation than observed in the absence of exogenous NTF2. Moreover, increasing the concentration of W7A-NTF2 above 1.5 μ M did not increase the efficiency of import further. Therefore, as well as binding xFxFG nucleoporin repeats less strongly, W7A-NTF2 also mediated the nuclear import of RanGDP less efficiently.

Figure 4. Fluorescence localization of NTF2 and W7A-NTF2 during RanGDP import. Fluorescein-labelled NTF2 or W7A-NTF2 (0.8 μ M) was incubated with permeabilized cells in the presence of an energy-regenerating system and, where indicated, with 2.3 μ M of RanGDP. NTF2 localization was analyzed after ten minutes by live confocal fluorescence microscopy. The addition of RanGDP enhanced accumulation of both wild-type NTF2 and W7A-NTF2 at the nuclear envelope; however, the effect on wild type NTF2 was significantly stronger than on W7A-NTF2.

Soluble Nsp1p xFxFG repeats inhibit both NTF2 binding to nuclear pores and nuclear import of RanGDP

We also examined the effect of the construct of Nsp1p containing 19 xFxFG repeats on both the localization of fluorescein-labelled wild-type NTF2 and on RanGDP nuclear import. As shown in Figure 7, the binding of wild-type NTF2 to the nuclear envelopes of digitonin-permeabilized HeLa cells in the presence of RanGDP and Nsp1p was much lower than that observed with RanGDP

Figure 5. Electron micrographs of sections of *Xenopus* oocytes after microinjection of colloidal gold coated with (a) wild-type or (b) W7A-NTF2. Gold coated with wild-type NTF2 is localized to the nucleoplasmic and cytoplasmic and also to the central channel of the NPC so that there is a continuous track of gold particles crossing the nuclear envelope at each NPC. Gold coated with W7A-NTF2 still showed binding to the nucleoplasmic and cytoplasmic faces of the NPCs, but showed substantially reduced binding to the central NPC channel (see also Table 3) so that now few NPCs showed a continuous track of gold particles crossing the nuclear membrane.

alone. Indeed, in the presence of the xFxFG repeat construct, the binding of fluorescein-labelled NTF2 to the nuclear envelope was comparable to that seen with the W7A mutant alone, demonstrating that the Nsp1p construct had competed effectively with the NPCs for NTF2 binding. Moreover, the addition of this xFxFG construct also inhibited the nuclear import of Ran. As illustrated in Figure 8, in permeabilized HeLa cells in the presence of reticulocyte lysate, addition of the Nsp1p construct to a concentration of 54 μ M significantly inhibited the import of fluorescein-labelled RanGDP, consistent with an interaction between xFxFG-containing nucleoporins and NTF2 being required for efficient nuclear import of Ran.

Discussion

Previous work has shown that NTF2 mediates the nuclear import of RanGDP (Smith *et al.*, 1998; Ribbeck *et al.*, 1998). Moreover, a direct interaction between these proteins is required, since Ran import is defective with mutants of NTF2 that do not bind RanGDP and with Ran mutants that do not bind NTF2. We have now used protein engineering, based on the crystal structure of wild-type

(Bullock *et al.*, 1996) and mutant (Clarkson *et al.*, 1997) NTF2 and the NTF2-RanGDP complex (Stewart *et al.*, 1998a) to produce a NTF2 mutant, W7A-NTF2, in which *in vitro* binding to xFxFG repeat-containing nucleoporins is reduced by a factor of at least an order of magnitude, while retaining binding to RanGDP (Figures 2 and 3). Light microscopy using fluorescein-labelled proteins (Figure 4) indicated that W7A-NTF2 bound to nuclear envelopes more weakly than wild-type and electron microscopy indicated that gold particles coated with W7A-NTF2 bound much less strongly to the central transport channel of the nuclear pore complexes in *Xenopus* oocytes. Moreover, in contrast to wild-type NTF2 (Smith *et al.*, 1998; Ribbeck *et al.*, 1998), W7A-NTF2 shows only weak stimulation of the nuclear import of fluorescently labelled RanGDP (Figure 6), providing direct evidence that the mechanism for NTF2-mediated Ran import involves an interaction of NTF2 with xFxFG repeat-containing nucleoporins.

Binding of NTF2 to NPCs

The reduced binding of W7A-NTF2 to nuclear envelopes relative to wild-type (Figure 4) and the

Table 3. Quantification of protein-labelled gold particles in electron micrographs of nuclear envelopes of micro-injected *Xenopus* oocytes (see Figure 5) as described by Feldher & Akin (1997)

Protein used to coat gold particles	Number of particles located in each NPC compartment		
	Cytoplasmic face	Pore channel	Nuclear face
Wild-type NTF2	502 (57 %)	115 (13 %)	268 (30 %)
W7A-NTF2	674 (46 %)	110 (8 %)	668 (46 %)

Figure 6. The NTF2-RanGDP interaction is required for Ran import. Nuclear import of fluorescein-labelled RanGDP ($1.5 \mu\text{M}$) was performed in permeabilized HeLa cells in the presence of an energy regenerating system and reticulocyte lysate that had been depleted of NTF2 (Ribbeck *et al.*, 1998). Uptake of fluorescein-Ran into the nuclei was analyzed after ten minutes by confocal fluorescence microscopy of living cells using the fluorescein channel. Addition of $0.75 \mu\text{M}$ wild-type NTF2 (dimer) efficiently restored Ran import activity, whereas addition of the W7A-NTF2 mutant even at approximately fourfold higher concentrations ($2.5 \mu\text{M}$) only weakly stimulated Ran import.

inhibition of the binding of wild-type NTF2 to nuclear envelopes by added xFxFG repeats (Figure 7) both indicate that NTF2's binding to NPCs is mediated through its interaction with the xFxFG repeat-containing nucleoporins such as Nsp1p and p62. However, both fluorescein-labelled material and NTF2-coated gold particles still showed some residual binding to the nuclear envelope. In the case of permeabilized cells, this residual binding was increased by the addition of RanGDP (Figure 4). There appeared to be more residual binding with the coated gold particles, but it is not straightforward to compare each method quantitatively. The residual binding present with the coated gold particles may have been due to the endogenous levels of Ran present in intact *Xenopus* oocytes which, as shown in Figure 4, would be expected to increase the level of NTF2 binding to the nuclear envelope. However, the residual binding seen with coated gold particles could also have derived, at least partially, from non-specific attachment of the gold particles to the periphery of the NPCs. Significantly the residual binding seen with gold particles coated with W7A-NTF2 was located mainly at the cytoplasmic and nucleoplasmic faces of the NPCs and was strikingly absent from the central transport channel of the pores where the majority of the xFxFG nucleoporins are thought to be located (Grote *et al.*, 1995).

The residual binding of both fluorescein-labelled W7A-NTF2 and W7A-NTF2-coated colloidal gold particles to NPCs could derive from several causes. For example, although both the bead-binding and

fluorescence assays (Figures 2 and 3) indicated that the interaction of NTF2 with xFxFG repeats was weaker in the W7A mutant, this interaction may not have been entirely ablated by this mutation. A residual binding of W7A-NTF2 to xFxFG repeats could account for the residual levels bound to NPCs seen by both light (Figure 4) and electron (Figure 5) microscopy and also for the observation that, although its Ran import activity was reduced, it was not completely eliminated. In addition, the environment in which NTF2 binding is occurring at the NPC may be rather different from that employed for solution binding studies. Although the effective concentration of xFxFG repeats within the pore is unknown, it is likely to be relatively high, since several nucleoporins contain these repeats, often in up to 20 copies per chain. Electron microscopy indicates that these repeats tend to be concentrated along the central channel (Grote *et al.*, 1995; Fahrenkrog *et al.*, 1998). If, as a first approximation, one assumes that there could be eight copies of ten different nucleoporins each containing 20 repeat cores located in the central channel occupying a cylinder $700 \text{ \AA}$ long and $300 \text{ \AA}$ in diameter, the effective xFxFG core concentration in the channel would be of the order of 50 mM . In addition, the labelled gold particles probably contained several NTF2 molecules and thus were also multidentate. Therefore, even quite weak interactions between W7A-NTF2 and the repeats could result in some binding occurring at the NPC, albeit considerably less than observed with wild-type NTF2. Alternatively, NTF2 could also be binding

Figure 7. Soluble Nsp1p xFxFG repeats compete with the nuclear pore for binding to NTF2. Fluorescein-labelled wild-type or W7A-NTF2 (1.75 μ M) was incubated in the presence of an energy-regenerating system and, where indicated, with 2.3 μ M of RanGDP. NTF2 localization was analysed after ten minutes by confocal fluorescence microscopy. The addition of 17 μ M Nsp1p construct containing 19 xFxFG repeats significantly reduced the binding of wild-type NTF2 to the nuclear envelope.

another, as yet unidentified nucleoporin. Although clearly further work will be required to define more precisely the basis of the residual binding of W7A-NTF2 to the nucleoplasmic face of NPCs, the loss of binding to the central channel is certainly consistent with the reduced binding to xFxFG nucleoporins observed *in vitro* (Figures 2 and 3).

The weak binding constant observed for the interaction between wild-type NTF2 and the xFxFG repeats of Nsp1p was consistent with NTF2 forming a number of transient attachments to xFxFG nucleoporins during nuclear Ran import. The operational binding constant calculated assuming no co-operation between sites was of the order of 3 μ M, but this probably overestimated the strength of the interaction because the locally high concentration of xFxFG cores could tend to facilitate rapid rebinding once NTF2 had dissociated. The on-rate of association between NTF2 and xFxFG repeats would be expected to be in the range 10^7 – 10^8 s⁻¹ (Berg & von Hippel, 1985), and so a 3 μ M binding constant would imply an off-rate of the order of 30–300 s⁻¹. Consequently a binding constant of this magnitude would imply that interactions between NTF2 and xFxFG nucleoporins would be transient and so ideally suited to allowing a rapid attachment and detachment of

Figure 8. Soluble Nsp1p inhibits nuclear import of RanGDP. Nuclear import of fluorescein-labelled RanGDP (1.5 μ M) was performed in permeabilized HeLa cells in the presence of an energy regenerating system and reticulocyte lysate. Uptake of fluorescein-Ran into the nuclei was analyzed after ten minutes by confocal fluorescence microscopy of these cells using the fluorescein channel. Without the addition of the Nsp1p construct containing 19 xFxFG repeats, fluorescein-labelled Ran accumulated in the nucleus and so gave a bright nuclear fluorescence signal, whereas addition of 54 μ M Nsp1p significantly inhibited nuclear import of Ran and resulted in a marked reduction of the nuclear fluorescence signal.

NTF2 to nucleoporins as it passed through a NPC. Thus, the K_d for the NTF2-xFxFG nucleoporin interaction is ideally suited for allowing the NTF2-RanGDP complex to translocate through NPCs by hopping from one nucleoporin to the next along the central channel.

Implications for nuclear protein import

The results obtained with W7A-NTF2 have demonstrated a direct involvement of the NTF2-xFxFG nucleoporin interaction in the nuclear import of RanGDP. This first direct demonstration of a functional interaction between a xFxFG nucleoporin and a transport factor raises the question of whether an analogous interaction is involved in other import pathways. Although other cargo molecules are imported using importin- β family member transport factors rather than NTF2 as carriers, there are a number of parallels between the import of Ran and that of other proteins. For example, interactions between members of the importin- β family and several xFxFG or GLFG repeat-containing nucleoporins have been identified by both *in vitro* binding assays and the *in vivo* yeast two-hybrid system (Radu *et al.*, 1995a,b; Rexach & Blobel, 1995; Bastos *et al.*, 1996; Iovine *et al.*, 1996; Shah *et al.*, 1998). Moreover, bacterially expressed constructs corresponding to

xFxFG repeat domains of several nucleoporins have been shown to exert a dominant negative effect on nucleocytoplasmic transport (Radu *et al.*, 1995b; Moroianu *et al.*, 1997; Shah & Forbes, 1998). A clear picture of the role of these sequence repeats in the import of nuclear transport factors and substrates has not yet emerged, although there appears to be a correlation between interactions with GLFG nucleoporins and export (Iovine & Wente, 1997), whereas interactions with xFxFG nucleoporins appear to be associated with import (Shah & Forbes, 1998). At least for trafficking involving importin- β , Rexach & Blobel (1995) have proposed that nuclear protein import could be mediated by cycles of attachment and detachment of cargo-carrying molecules to these repeats. The extent to which the interaction of carrier molecules of the importin- β family with nucleoporins resembles that between NTF2 and nucleoporins is not completely clear. However, importin- β (or the importin α/β heterodimer) appears to only interact with some xFxFG nucleoporins and not with others (see, for example, Percipalle *et al.*, 1997; Shah *et al.*, 1998), whereas NTF2 appears to interact more generally with xFxFG nucleoporins (Paschal & Gerace, 1995; Clarkson *et al.*, 1996, 1997). Although blot overlays (Nehrbass & Blobel, 1996) indicated that NTF2 binds to both xFxFG and GLFG repeat-containing nucleoporins, yeast two-hybrid assays (Clarkson *et al.*, 1997) showed only an interaction between NTF2 and xFxFG repeat-containing nucleoporins and did not detect an interaction between NTF2 and GLFG-repeat containing nucleoporins. Thus, whereas NTF2 is probably binding to the xFxFG cores of these repeats (Clarkson *et al.*, 1997), it may be that the importin α/β heterodimer is binding instead to some of the linkers. Moreover, it could be that the interaction between nucleoporins and importin- β could be indirect and involve both importin- β and the nucleoporins binding an additional protein. Furthermore, although addition of bacterially expressed constructs containing the repeat regions of xFxFG nucleoporins can act as a dominant negative inhibitor of nuclear protein import (Radu *et al.*, 1995a; Moroianu *et al.*, 1997; Shah & Forbes, 1998), it is not clear that this effect is mediated directly through an interaction with importin- β . Indeed added xFxFG repeats could, for example, inhibit nuclear protein import by sequestering NTF2 and thereby inhibiting RanGDP's nuclear import and associated recharging with GTP by RCC1. Clearly further experimental work will be needed to resolve this point and, in this context, unfortunately it has not yet been possible to generate importin- β mutants analogous to W7A-NTF2 in which the binding to xFxFG nucleoporins was reduced while retaining their other interactions associated with trafficking. However, the general similarity between the NTF2 and importin- β pathways certainly lends plausibility to the idea of the translocation through NPCs of an importin α/β substrate complex being mediated by a

series of weak interactions with repeat-containing nucleoporins.

In summary, W7A-NTF2 retains its affinity for RanGDP, but has reduced affinity for xFxFG nucleoporins *in vitro* and binds less strongly to NPCs. Because the structure of W7A-NTF2 is very similar to that of the wild-type protein, a binding site for xFxFG nucleoporins on NTF2 is probably at or near Trp7. Indeed it is likely that the side-chain of residue 7 participates directly in the interaction with the hydrophobic residues in the cores of the xFxFG repeats and this would provide a rationale for why so many nucleoporin repeats are rich in hydrophobic residues such as phenylalanine. W7A-NTF2 stimulates the nuclear import of RanGDP less effectively than wild-type NTF2, and so provides direct evidence for the involvement of an interaction between NTF2 and xFxFG nucleoporins in this process.

Materials and Methods

Molecular biology

The W7A mutant of murine NTF2 was made by using PCR-based site-specific mutagenesis as described (Clarkson *et al.*, 1997; Stewart *et al.*, 1998b). After cloning into the phage T7-based expression vector, pET15b, the inserted DNA was sequenced to ensure that Trp7 was mutated to Ala7 and that no other mutations were present in the construct. W7A-NTF2 protein was expressed in *Escherichia coli* strain BL21(DE3) at 34°C overnight without induction. Protein was purified by DE52 ion-exchange chromatography followed by gel-filtration on an S100 column using the protocol described previously for wild-type NTF2 (Kent *et al.*, 1996). The purified protein was over 95% pure by SDS-PAGE using Coomassie staining (Laemmli, 1970).

Bead-binding assays

Solution binding assays using either wild-type or W7A NTF2 were performed as described (Clarkson *et al.*, 1997) with minor modifications. NTF2 was coupled to CNBr-Sepharose beads at a concentration of 3.6 mg/ml; and binding and washing were carried out in PBS + 0.1% (v/v) Tween-20 to reduce non-specific interactions. Ran, the repeat domain of Nsp1p containing 19 xFxFG repeats, and the repeat domain of p62 containing 11 xFxFG repeats were all obtained by expression in *E. coli*, and purified as described (Buss & Stewart, 1995; Clarkson *et al.*, 1996). The p62 protein was pre-treated by incubation with ovalbumin-coupled Sepharose beads to remove the sub-population of molecules which appeared to bind non-specifically to ovalbumin and could not be washed out. In order to determine K_d values, Nsp1p was coupled to CNBr-Sepharose beads at a concentration of approximately 3 mg/ml. Binding of wild-type or W7A-NTF2 was carried out as before in PBS + 0.1% Tween-20, but the washing steps were omitted because they reduced the effective concentration of NTF2 in solution. The amount of NTF2 bound to beads was visualized by digitizing the SDS-PAGE protein gel using either the Kodak Digital Science system or a Molecular Dynamics Personal Densitometer SI and quantitating using Image-

Quant (Molecular Dynamics). The amount of NTF2 occluded in the bead pellet was estimated using ovalbumin-coupled Sepharose beads. Occlusion of NTF2 in ovalbumin beads was linear with respect to free NTF2 concentration and subtracting this line from Nsp1p-bead data resulted in binding curves shown (Figure 3(b)). It was not possible to couple the p62 construct to CNBr-Sepharose beads and so K_d values for the interaction between p62 and NTF2 were determined using either wild-type or W7A-NTF2 coupled Sepharose beads and adding soluble p62 which had been pretreated with ovalbumin-Sepharose beads.

Fluorescence assays

Wild-type rat NTF2 contains three tryptophan residues, which give a fluorescence emission maximum at 340 nm when excited at 290 nm. We developed an assay based on the observation that this fluorescence was increased when NTF2 binds xFxFG repeat-containing proteins. The Nsp1p construct contained no tryptophan or tyrosine residues and showed less than 1/2000 the intrinsic fluorescence of wild-type NTF2 when excited at 290 nm. Therefore, we could be confident that the fluorescence change observed in our assay was due to the interaction between the xFxFG repeats and NTF2. Titrating the Nsp1p protein into a fixed quantity of NTF2 lead to an increase in fluorescence which, since Nsp1p had negligible intrinsic fluorescence, was attributed to the increase in NTF2 tryptophan fluorescence due to binding of Nsp1p (probably as a result of shielding from the solvent). Measurements of the intrinsic tryptophan fluorescence of NTF2 at 340 nm after excitation at 290 nm were carried out using a Perkin Elmer LS50B fluorimeter and FL Winlab software. Using slit widths of 2.5 nm and 5 nm for excitation and emission, respectively, the intrinsic fluorescence of both W7A and wild-type NTF2 varied linearly with concentration demonstrating that there were no artefacts due to the inner filter effect. A stock solution of Nsp1p protein was added serially in 2 μ l aliquots to 2.3 μ M W7A and 0.6 μ M wild-type NTF2 (as assayed by absorbance at 280 nm) in an initial volume of 400 μ l of PBS containing 2 mM $MgCl_2$. Appropriate concentrations of mutant and wild-type NTF2 were used to obtain sufficient signal from the tryptophan fluorescence. Fluorescence measurements were integrated for two seconds, repeated three times, averaged and corrected for volume for each point. Binding isotherms were generated using a simple least-squares curve-fitting algorithm and Graft software.

Fluorescein labelling

NTF2 and W7A-NTF2 were labelled essentially as described (Ribbeck *et al.*, 1998) in 50 mM Tris-buffer (pH 7.5) containing 200 mM NaCl, 5 mM Mg acetate. An equimolar amount of fluorescein-5' O-maleimide (dissolved in dimethyl formamide) was added and the reaction was carried out on ice for four hours. Non-incorporated label was removed by gel filtration on a NAP 5 column (Pharmacia) equilibrated with 50 mM Tris (pH 7.5), 200 mM NaCl, 5 mM Mg acetate, 250 mM sucrose.

Electron microscopy

Xenopus laevis were purchased from Xenopus I (Ann Arbor, MI, USA) and maintained in filtered artificial

pond water. Stage VI oocytes, approximately 1.2 mm in diameter, were obtained from mature females and had well-delineated animal and vegetal hemispheres. Colloidal gold particles 20-50 Å in diameter were coated with wild-type NTF2 or the W7A mutant and microinjected into manually defolliculated stage VI oocytes as described (Feldherr *et al.*, 1998) using a Narishige micro-manipulator and 10-15 μ m tip diameter micropipettes. After approximately 60 minutes the injected oocytes were fixed with glutaraldehyde followed by OsO_4 , dehydrated and embedded as described (Dworetzky & Feldherr, 1988). Sections were examined in a JEOL 100CX electron microscope.

RanGDP nuclear import assay

The nuclear import of fluorescein-labelled Ran in permeabilized Hela cells was carried out in suspension at 19°C essentially as described (Ribbeck *et al.*, 1998). The import buffer contained 2 mg/ml nucleoplasmin core (to block non-specific binding), 20 mM Hepes/KOH (pH 7.5), 120 mM potassium acetate, 5 mM Mg acetate, 250 mM sucrose, 0.5 mM EGTA. The energy-regenerating system consisted of 0.5 mM GTP, 0.5 mM ATP, 10 mM creatine phosphate and 50 mg/ml creatine kinase.

X-ray crystallography

W7A-NTF2 crystals with $P2_1$ symmetry were obtained by vapour diffusion using 8 μ l hanging drops containing approximately 1.5 mg/ml protein and half-strength mother liquor at 18°C against a mother liquor of 1.32 M ammonium sulphate, 3% (v/v) isopropanol, 100 mM Tris-HCl (pH 7.5). After 15 hours, the drops were microseeded using fragments of old W7A crystals. After three days the crystals were fully grown. A plate crystal of dimensions 0.2 mm $\times$ 0.1 mm was transferred to cryoprotectant (mother liquor plus 20% (v/v) glycerol) for less than one minute and was flash-frozen at 100 K. A native data set was collected at beamline 5.2 at ELETTRA using synchrotron radiation of wavelength 1.2 Å and a Mar345 detector and was reduced using MOSFLM and SCALA (CCP4, 1994). Table 1 presents the data collection statistics. It was possible to index the data set as having either $C222_1$ or $P2_1$ symmetry. We chose to process the pattern using $P2_1$ symmetry because we had obtained $P2_1$ crystals with similar lattice constants using another NTF2 mutant (unpublished data) and so thought it prudent not to risk introducing spurious higher symmetry. Molecular replacement using a 2.3 Å resolution wild-type NTF2 structure (Bullock *et al.*, 1996; PDB accession code 1OUN) and AMORE (CCP4, 1994) produced a final R -factor of 20.8% and R_{free} of 24.0%. We used O (Jones *et al.*, 1991) for chain-tracing and model building and REFMAC (CCP4, 1994) for refinement.

Protein Data Bank accession number

The co-ordinates of the W7A-NTF2 structure have been deposited in the Brookhaven protein data base with accession code 1qma.

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