The human homologue of yeast CRM1 is in a dynamic subcomplex with CAN/Nup214 and a novel nuclear pore component Nup88

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The oncogenic nucleoporin CAN/Nup214 is essential in vertebrate cells. Its depletion results in defective nuclear protein import, inhibition of messenger RNA export and cell cycle arrest. We recently found that CAN associates with proteins of 88 and 112 kDa, which we have now cloned and characterized. The 88 kDa protein is a novel nuclear pore complex (NPC) component, which we have named Nup88. Depletion of CAN from the NPC results in concomitant loss of Nup88, indicating that the localization of Nup88 to the NPC is dependent on CAN binding. The 112 kDa protein is the human homologue of yeast CRM1, a protein known to be required for maintenance of correct chromosome structure. This human CRM1 (hCRM1) localized to the NPC as well as to the nucleoplasm. Nuclear overexpression of the FG-repeat region of CAN, containing its hCRM1-interaction domain, resulted in depletion of hCRM1 from the NPC. In CAN-/- mouse embryos lacking CAN, hCRM1 remained in the nuclear envelope, suggesting that this protein can also bind to other repeat-containing nucleoporins. Lastly, hCRM1 shares a domain of significant homology with importin-β, a cytoplasmic transport factor that interacts with nucleoporin repeat regions. We propose that hCRM1 is a soluble nuclear transport factor that interacts with the NPC.

Keywords: CAN/Nup214/CSE1/importin-β/nuclear pore complex/nucleocytoplasmic transport

Introduction

The nuclear pore complex (NPC) is a ~125 MDa complex embedded in the nuclear envelope (NE) that mediates bidirectional nucleocytoplasmic traffic in eukaryotic cells (recently reviewed by Panté and Aebersold, 1994, 1996; Simons and Hurt, 1995; Görlich and Mattaj, 1996).

Although more than 30 NPC components have been isolated in both yeast and vertebrates, the interactions between the NPC and trafficking macromolecules are only recently beginning to be understood. The import of nuclear localization signal (NLS) carrying proteins into the nucleus is mediated by a heterodimeric receptor complex. The smaller subunit of this complex, named importin-α, NR-α or karyopherin-α, binds directly to the NLS (Adam and Gerace, 1991; Adam and Adam, 1994; Görlich et al., 1994). The larger subunit, named p97, importin-β, NR-β or karyopherin-β, is thought to mediate docking to the NPC. Importin-β binds to several repeat-containing nucleoporins in vitro, and certain nucleoporin repeats may act as the docking sites for the NLS import complex (Chi et al., 1995; Görlich et al., 1995b; Moroianu et al., 1995; Rada et al., 1995b). After docking, the import complex translocates though the central pore of the NPC, and the import substrate is released into the nucleoplasm in an energy-dependent manner, requiring the Ras-like GTPase Ran/TC4 (Melchior et al., 1993; Moore and Blobel, 1993; for a recent review, see Schlesinger, 1996).

Export of proteins and ribonucleoproteins (RNPs) from the nucleus is also an active process that uses some of the same factors involved in protein import, notably Ran/TC4 (Schlesinger et al., 1995) and importin-α (Görlich et al., 1996).

Within the NPC, several proteins interact in a genetic or physical manner. Co-purification studies in yeast showed that the nucleoporin Nsp1 forms one complex with the nucleoporins Nup49, Nup57 and Nup96 (Grandi et al., 1993), and forms a separate complex with Nup82 (Grandi et al., 1995). Other yeast proteins that physically interact include Srpl, Nup1 and Nup2 (Belanger et al., 1994). Interestingly, Srpl is the yeast homologue of importin-α. Recently, a yeast complex has been identified that includes nucleoporins Nup84, Nup120, Nup85 and also Sec13, which is involved in the transport of proteins from the endoplasmic reticulum to the Golgi apparatus (Siniossoglou et al., 1996). In higher eukaryotes, two protein subcomplexes have been identified, one containing nucleoporin p62, complexed with proteins of 58, 54 and 45 kDa (Panté et al., 1994; Guan et al., 1995), and the second containing p250, associated with a protein of 75 kDa (Panté et al., 1994). P62 is the metazoan homologue of yeast Nsp1, and p250 is probably identical to CAN/Nup214.

The CAN protein was originally identified through its involvement in two types of acute myeloid or undifferentiated leukemia (von Lindern et al., 1992a,b). We have recently developed an in vivo approach to study the consequences of CAN depletion in knock-out mouse embryos. Using this approach, we found that the absence of CAN leads to simultaneous defects in nucleocytoplasmic transport and in cell cycle progression (van Deursen et al., 1996). Previously, we identified a new CAN-containing complex that included proteins of 88 and 112 kDa (Fornerod et al., 1996). The central region of CAN associates with the 88 kDa protein, most likely through coiled-coil interactions, whereas the 112 kDa protein interacts with part of CAN’s nucleoporin-specific...
Fig. 1. Immunopurification of CAN-associating proteins. (A) Proteins immunoprecipitated with monoclonal antibody 12CA5 from cell lines expressing an HA1-tagged version of CAN (TTB6 and TTD2), DEK-CAN (C4322), and the parental cell line (H7A-1), separated electrophoretically on 6% polyacrylamide gels and visualized with silver staining. The positions of CAN and DEK-CAN, as well as those of the coprecipitating proteins CC112 and CC88, are indicated by arrows. A molecular weight standard is indicated on the right. IgG-H, immunoglobulin heavy chain. (B) An aliquot (0.1%) of the large-scale immunopurification of CAN-associating proteins from the TTD2 cell line, run on a 6% polyacrylamide gel and silver stained. Arrows indicate the 112 and 88 kDa copurifying proteins, and molecular weight markers are shown in an adjacent lane (right panel). As a reference, HA1–CAN was coprecipitated from [3H]leucine-labeled TTD2 cells (left panel). The position of CAN and coprecipitating proteins CC112, CC88 and CC66 are indicated on the left.

way we generated C4322, expressing HA1-tagged DEK-CAN (Fornerod et al., 1995). Only the 112 kDa protein coprecipitated from this cell line. To visualize the coprecipitating proteins on a silver-stained gel (Figure 1A) we needed at least 107 cells per immunoprecipitation. For micro-amino acid sequence analysis, we scaled up the immunoprecipitation ~1000-fold, using cell line TTD2 (see Materials and methods). Proteins from the preparation were separated by SDS–PAGE (Figure 1B), and the 88 and 112 kDa protein bands were excised from the gel. Quantities of a coprecipitating protein of 66 kDa (CC66: Fornerod et al., 1996) were insufficient for further analysis. Gel slices containing 82 and 48 pmol of CC88 and CC112 respectively were digested with trypsin in situ, and tryptic peptides were eluted, purified by preparative HPLC and then sequenced from the N-termini. Two sequences of 16 amino acids were obtained from CC88, and CC112 yielded one sequence of seven amino acids (Table I). None of these sequences showed significant homology to known proteins; however, the two peptides derived from CC88 matched an uncharacterized human cDNA in the dBEST database (IMAGE clone 179414, Genbank Accession number H50498). Similarly, the peptide derived from CC112 matched a cDNA in the TIGR database (Clone HTTEU26, Human Genome Science, Rockville, MD). Interestingly, the amino acid sequence of the putative reading frame of this cDNA clone showed significant homology to a yeast protein of 115 kDa, named CRM1. To determine whether these cDNAs were indeed derived from mRNAs encoding CC88 and CC112, full-length cDNA sequences were obtained from a human placenta cDNA library using clones 179414 and HTTEU26 as probes.

Sequence analysis of hCRM1 and Nup88

The complete cDNA putatively encoding CC112 had an open reading frame of 1071 amino acids and encoded a protein with a predicted molecular mass of 123 kDa (Figure 2A). This open reading frame showed high homology to Saccharomyces cerevisiae CRM1 (47% identity, 67% similarity) and to the Schizosaccharomyces pombe homologue CRM1 + (52% identity, 69% similarity). We therefore named this protein hCRM1 (human CRM1). Further database searches revealed that the N-terminus of hCRM1 shared significant homology to the N-terminus of importin-β (Figure 2B). Importin-β is part of the nuclear protein import receptor and can bind CAN in vitro (Radu et al., 1995). In addition we found that a group of largely uncharacterized yeast and vertebrate proteins of similar size (110–120 kDa) shared this homology domain, that we propose to name the CRIME domain (CRM1, Importinβ, Etcetera). The sequence divergence within the group was calculated according to Sneath and Sokal (1973) (Figure 2C).

The complete cDNA thought to encode CC88 had an open reading frame of 741 amino acids and a predicted molecular mass of 85 kDa (Figure 3A). Because an unrelated protein named Nup85 already exists, we have named this protein Nup88. Database searches revealed no significant homology of Nup88 to known proteins. However, the C-terminal sequences of Nup88 are predicted to form a coiled-coil (Lupas et al., 1991; Figure 3B), an interaction domain often found in NPC proteins.

Results

Purification of CAN coprecipitating proteins

We showed recently that two proteins specifically co-immunoprecipitate with CAN, one of 88 kDa (CC88) and one of 112 kDa (CC112) (Fornerod et al., 1996). To coprecipitate sufficient quantities of the 112 and 88 kDa proteins for micro-amino acid sequence analysis, we created stable cell lines that express an HA1-tagged CAN protein. To avoid toxic effects of high CAN expression (Fornerod et al., 1995), we made use of the Tet-VP16 system (Gossen and Bujard, 1992) to repress HA1–CAN during the transient phases of transfection. Two independent, stably transfected cell clones, TTB6 and TTD2, were analyzed for proteins that coprecipitate with CAN. As shown in Figure 1A, both cell lines coprecipitated the expected 88 and 112 kDa proteins. In much the same

repeat region. Identification of these proteins by molecular cloning could improve our understanding of the function of CAN in the NPC. In addition, CC112 might be important in the leukemic process associated with DEK–CAN and SET–CAN, because it interacts with these leukaemia-specific fusion proteins (Fornerod et al., 1996).

Here, we report the cloning and characterization of these 88 and 112 kDa proteins. The 88 kDa protein is a new nuclear pore component that we name Nup88. The 112 kDa protein is the human homologue of yeast CRM1 and is located at the NPC and nucleus. We provide evidence that the human CRM1 protein binds multiple NPC components and moves between the nuclear pore and the nucleoplasm. We also identify a group of proteins that includes hCRM1, yeast CRM1 and importin-β, which may constitute a novel family of NPC-interacting transport factors.

808
Table 1. Amino acid sequences of tryptic peptides derived from Nup88 and hCRM1

<table>
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<th>Protein</th>
<th>Peptide/cDNA</th>
<th>Amino acid sequence</th>
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<tr>
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<td>CC88 peptide 30</td>
<td>GPSSGGEPPALS(SQYQR)</td>
</tr>
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<td></td>
<td>cDNA IMAGE: 174414</td>
<td>RGPSGGGEPPALS S QYYR</td>
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<tr>
<td></td>
<td>CC88 peptide 66pep20</td>
<td>-XQPTEEKPA(S)SGG(SG)PG(K)</td>
</tr>
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<td>cDNA IMAGE: 174414</td>
<td>KNSQPTEEKPA S S S L P S</td>
</tr>
<tr>
<td></td>
<td>CC112 peptide 66pep20</td>
<td>-LISGWV9(R)</td>
</tr>
<tr>
<td></td>
<td>cDNA TIGR HTTEU26</td>
<td>K1ISGWVRS</td>
</tr>
</tbody>
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*Compared with virtual translations of expressed sequence tags found in computer databases. Trypsin hydrolyzes peptide bonds at the C terminal side of lysine (K) or arginine (R).*

**Fig. 2.** Amino acid sequence of hCRM1. (A) Comparison between hCRM1, *Saccharomyces* CRM1 (Adachi and Yamagata, 1989; Toza et al., 1992), and *S. pombe* CRM1 (Yoda et al., 1992). Identical and similar amino acids are boxed in black and gray, respectively. The broken lines above the sequence denote the N terminal homology domain and an asterisk indicates the conserved tryptophan. A bar indicates the position of peptide 66pep20. The amino acid sequences between the arrows were used to raise antibodies against the protein. (B) Comparison between hCRM1 amino acids 78-154 and similar N-terminal regions of *Saccharomyces* CRM1, *C. elegans* (Nud1, 1997b), *Y. enterocolitica* (P40099), *P. aeruginosa* (P38240), *E. coli* (P36937), *H. pylori* (P54684), and *C. elegans* (P36937). (C) Amino acid sequences were calculated using the program Clustal W with a gap penalty of 10 and a gap extension penalty of 0.05. (D) A dot diagram representing the sequence relationships between CRM1 domain proteins. Relationships were calculated using the UP-GMA algorithm (Sneath and Sokal, 1973) and were based on complete amino acid sequences.

**Interaction of Nup88 and hCRM1 with CAN**

To further study the Nup88 and hCRM1 proteins, and to confirm their interaction with CAN, we produced rabbit polyclonal antisera against amino acids 509-741 of Nup88 and amino acids 805-1071 of hCRM1 (Figures 2 and 3). These regions excluded the peptide sequences used to identify the Nup88 and hCRM1 cDNAs.

To confirm that isolated cDNA sequences indeed encoded the 88 and 112 kDa CAN-associating proteins, we tested whether affinity-purified α-Nup88 and α-hCRM1 antisera could detect these proteins in CAN immunoprecipitates. Using IP-Western blot analysis (Figure 4A), we found that the 112 kDa protein that was immunopurified from TTD2 or C4322 cells was recognized by
The absorption of light by all (1.66) the photosynthetic units and the energy transfer within the photosynthetic apparatus of photosystem II, which drive the electron transport chain. The transient and dark reactions of the carbon cycle (photosynthesis) provide the basic building blocks of life on Earth. A complete understanding of the photosynthetic process is essential for developing sustainable energy sources and mitigating climate change. By improving the efficiency of photosynthesis in crop plants, we can enhance crop yields and reduce the carbon footprint of agriculture. Additionally, understanding the fundamental principles of photosynthesis can inform the development of new materials and technologies for energy conversion and storage.
NCp is a Ca2+ dependent negative feedback mechanism that regulates the expression of NKB in the hippocampus. The NKB-ergic system is involved in the modulation of synaptic plasticity and learning processes. The results of this study suggest that the NKB-ergic system plays a crucial role in the modulation of synaptic plasticity and learning processes, and that the NCp is a negative feedback mechanism that regulates the expression of NKB in the hippocampus.
situation, or if there is trafficking of hCRM1 between these cellular compartments.

In previous studies, we found that the C-terminal part of CAN is located in the nucleus when expressed by itself (Fornerod et al., 1995). Moreover, this part of CAN includes the hCRM1-binding domain (Fornerod et al., 1996). If hCRM1 moves between the nuclear pore and the nucleoplasm, the presence in the nucleus of the CAN domain for hCRM1 binding might disturb hCRM1 intracellular routing. We therefore expressed the hCRM1-binding domain of CAN (the C-terminal amino acids 1864–2090) transiently in HtTA-1 cells. By using indirect immunofluorescence, we could detect the transfected protein with monoclonal 12CA5 (Figure 7A), while, in the same cells, we could monitor endogenous hCRM1 localization by using α-hCRM1 antibodies (Figure 7B). Expression of C-terminal CAN caused hCRM1 accumulation in the nucleus and its disappearance from the nuclear envelope, as we could verify by a double-labeling experiment with monoclonal 414, that specifically stains the nuclear envelope (data not shown). These results suggest that the hCRM1-binding domain of CAN is able to titrate hCRM1 from the NPC, and that the presence of hCRM1 at both the nuclear pore and in the nucleoplasm is a result of a dynamic exchange. However, if the turn-over time of hCRM1 is relatively short, nuclear hCRM1 accumulation could also be explained by newly synthesized hCRM1 becoming trapped in the nucleoplasm by C-terminal CAN.

We therefore determined the half-life of hCRM1 by pulse-chase experiments, and found it to be ~24 h (data not shown). This means that at least part of the hCRM1 protein that accumulates in the nucleus was originally located at the nuclear envelope.

As stated earlier, hCRM1 is present very prominently in the nucleoli of CAN-depleted embryos. Such accumulation could indicate that the phenotypic effects of CAN-depletion include disruption of processes within the nucleolus. In an attempt to mimic such disturbances, we cultured HtTA-1 cells for 45 min in the presence of 0.04 μg/ml actinomycin D, a compound which, at this concentration, specifically inhibits RNA polymerase I-dependent transcription (Perry and Kelley, 1970). Cells cultured in the presence of actinomycin D distinctly accumulated hCRM1 in their nucleoli (Figure 7D). Higher concentrations of actinomycin D (5 μg/ml), which also affect RNA polymerase II-dependent transcription, had the same effect on hCRM1 localization (not shown). These results suggest that the nucleolus may be part of normal hCRM1 routing, and encourage the design of studies into the role of hCRM1 in nucleolar function and nucleolar/NPC trafficking.

**Discussion**

The oncoprotein nucleoporin CAN/Nup214 forms an NPC subcomplex with proteins of 88 and 112 kDa (Fornerod
et al., 1996). We immunopurified these two proteins and cloned their cDNAs via peptide sequencing.

The 88 kDa protein is a novel nuclear pore component, which we have named Nup88. This protein may be identical to p75, a protein previously shown to copurify with CAN from rat liver extracts (Panté et al., 1994), although the difference in molecular weight seems to be considerable. Nup88 has no sequence homology to known proteins, but its C-terminus contains sequences that are predicted to form a coiled-coil domain. Predicted coiled-coil regions have been found in several other nuclear pore proteins, including CAN (for a review, see Panté and Aebi, 1994), and are thought to mediate interactions within NPC subcomplexes. Previously, we showed that mutations in CAN's coiled-coiled regions inhibit CAN interaction with the 88 kDa protein identified here as Nup88 (Fornerod et al., 1996). This result suggests that the Nup88–CAN interaction is coiled-coil-mediated. The position of the coiled-coil region of Nup88 is similar to that of *S. cerevisiae* Nup82p, a protein that, if mutated, causes mRNA export defects (Hurwitz and Blobel, 1995), as does mutation of CAN (van Deursen et al., 1996). However, the sequence homology between these proteins is marginal (data not shown), and it remains to be determined whether Nup88 could be the functional homologue of yeast Nup82p.

CAV-1 mouse embryos that lack CAN have no detectable Nup88 at their nuclear envelopes. Therefore, the presence of Nup88 at the nuclear pore depends on its physical interaction with CAN. This implies that the phenotypic effects of CAN elimination, which include G2 arrest and changes in nucleocytoplasmic trafficking (van Deursen et al., 1996), may in part be caused by Nup88 depletion from the NPC.

Surprisingly, the 112 kDa protein appeared to be the human homologue of *S. cerevisiae* and *S. pombe* CRM1, proteins not previously implicated in nucleocytoplasmic transport. The *S. pombe crm1* (chromosome region maintenance) gene was first identified as a mutated gene in certain cold-sensitive strains that display deformed chromosomes at the restrictive temperature (Adachi and Yanagida, 1989). Furthermore, mutations in the *crm1* gene cause deregulation of the transcription factor *pap1* (the budding yeast homologue of human API) (Toda et al., 1992), and can lead to multidrug resistance (Nishi et al., 1994; Turi et al., 1994). Mutations in yeast genes involved in nucleocytoplasmic trafficking can, apart from transport defects, lead to similar pleiotropic effects, as illustrated by the yeast homologue of the GTPase Ran/TC4, and its exchange factor RCC1 (Forrester et al., 1992; Kadowaki et al., 1993), and by Nup85p (Goldstein et al., 1996). It is therefore conceivable that a transport defect may be responsible for the *crm1* phenotype. The yeast CRM1 proteins have been localized to the nucleus and are particularly prominent at the nuclear periphery (Adachi and Yanagida, 1989). This, together with the high homology between yeast and human CRM1, suggests that also
in yeast, CRM1 may strongly associate with repeat-containing nucleoporins.

The hCRM1 protein is identical to the 112 kDa protein that interacts with DEK–CAN and SET–CAN, two nuclear fusion proteins associated with acute myeloid and undifferentiated leukemia, respectively (Fornerod et al., 1996). Because hCRM1 is not related to any proteins known to be involved in oncogenic transformation, its possible role in leukemogenesis remains to be determined. However, hCRM1 could be part of a novel pathway, via which nuclear pore components contribute to leukemogenesis.

Is hCRM1 a novel transport factor?

Several lines of evidence support the idea that hCRM1 could be a transport factor that interacts dynamically with the NPC.

First, the dual subcellular localization of hCRM1 to the nucleus and to the NPC suggests that this protein can travel between the two compartments. To test this, we overexpressed the hCRM1-binding domain of CAN, which is located in the nucleus and not at the NPC. If hCRM1 binds permanently to CAN, expression of its binding domain in the nucleus would have no effect. If, on the other hand, hCRM1 is released periodically from the NPC into the nucleus, the presence of an excess binding domain could sequester the hCRM1 in the nucleoplasm and lead to a gradual disappearance of hCRM1 from the nuclear envelope. We found that, under these conditions, hCRM1 was completely absent from the nuclear envelope and was only present in the nucleoplasm. Since we showed that the half-life of hCRM1 is long, this suggests that hCRM1 can move from the NPC to the nucleoplasm. In addition, we have shown that repression of RNA polymerase I-dependent transcription causes accumulation of hCRM1 in the nucleolus. Although we do not understand the mechanism causing this effect, it does suggest that hCRM1 routing involves the nucleolus.

Second, the nuclear envelopes of cells from CAN-depleted mouse embryos contain hCRM1. This suggests that hCRM1 can bind to NPC components other than CAN. We previously demonstrated that hCRM1 interacts with the C-terminal half of CAN’s nucleoporin repeat region (Fornerod et al., 1996). This repeat of CAN has significant homology to repeats of several other nucleoporins, including Nup98, Nup153 and p62. Thus, hCRM1 may interact with repeat regions of these, or other yet unknown, vertebrate nucleoporins. In agreement with this, we observed that hCRM1 is present at the nuclear as well as the cytoplasmic face of the NPC, while CAN is only present at the cytoplasmic side (Kraemer et al., 1994). Therefore, Nup98 and Nup153 are good candidates to mediate additional nuclear NPC association of hCRM1, as both reside at the nuclear face of the NPC (Stukegawa and Blobel, 1993; Radu et al., 1995b).

Third, hCRM1 shares a region of significant homology with importin-β. This factor interacts physically with nucleoporin-specific repeat regions (Moroianu et al., 1995; Radu et al., 1995b) and can bind CAN in ligand blot assays (Radu et al., 1995a). This suggests that hCRM1 and importin-β may interact with the NPC by the same mechanism. Importin-β forms part of a cytoplasmic transport complex that mediates protein import into the nucleus. Two molecular mechanisms have been proposed for the translocation of this protein–import complex through the NPC (Rexach and Blobel, 1995; Görlich and Mattaj, 1996; Nehrbaß and Blobel, 1996). Both models propose a stepwise binding and release of the importin-β component of the complex to and from nucleoporin repeats. Because the different nucleoporins localize to specific sites along the NPC, the transport direction of the complex is proposed to be established via an increased binding affinity of importin-β for nucleoporin repeats towards the nucleus. Following these models, the more cytoplasmically located CAN would have a relatively weak affinity for importin-β, which is in agreement with its absence in our CAN co-immunoprecipitation experiments. In contrast, hCRM1 appears to have a high affinity for CAN. This suggests that if hCRM1 interacts with nucleoporin repeats in an importin-β-like fashion, it could move in the opposite direction, i.e. from the nucleus to the cytoplasmic face of the NPC.

In addition to importin-β and its yeast homologue Kap95p, we found nine other proteins that share the N-terminal CRM1 domain. The majority of these proteins came from hypothetical open reading frames identified as part of the S.cerevisiae genome sequencing project. However, CSE1 has been identified as an essential yeast protein, and its mutation results in a chromosome segregation defect (Xiao et al., 1993). Moreover, it was reported that the cse1 phenotype can be suppressed by high expression of Smr1 (Belanger et al., 1994), the yeast importin-α homologue that interacts with the nucleoporins Nup1 and Nup2. Thus, CSE1 is the third otherwise unrelated protein that shares the N-terminal domain and is implicated in NPC interaction. Therefore, this N-terminal homology domain may define a new group of NPC-interacting transport factors, and it will be interesting to test whether it is this domain that mediates interaction with nucleoporin repeat sequences.

Materials and methods

Cell culture and transfection

HTA-1 cells (Gossen and Bujard, 1992) were cultured as described (Fornerod et al., 1995a). In some experiments, antitoxicin D (Boehringer Mannheim, Indianapolis, IN) or cycloheximide (Sigma, St Louis, MO) were added to the culture medium. Cell lines TTD2 and TT6B, which express HA1–CAN under the control of a tetracycline-dependent promoter, were created by co-transfecting HTA-1 cells with Smp linearized plasmid pHAl–CAN (Fornerod et al., 1995) and 5c1 linearized pHαPuro at a molar ratio of 20:1. Puramycin-resistant clones were selected as described (Fornerod et al., 1995). TTD2 expressed HA1–CAN predominantly in the nuclear envelope in the absence of tetracycline. Under these conditions, the cell line showed normal growth characteristics for multiple passages. The HA1–DEK–CAN-expressing cell line C4322 has been described previously, as has HA1–t transfection (Fornerod et al., 1995). Plusmid pHα1–Nup88(368–741) was created by placing sequences encoding two copies of the influenza virus HA1 tag (Fornerod et al., 1995) at the 5' side of codons 368–741 of the Nup88 cDNA.

Immunopurification

Approximately 10^10 TTD2 cells cultured on 500 15-cm dishes were rinsed once with phosphate-buffered saline (PBS), scraped in PBS and pelleted at 2000 g at 50 ml tubes for 10 min at 4°C. Cell pellets, in total weighing 40 g (wet weight) were frozen at −80°C until further processing. TTD2 cell aliquots (2 g each) were transferred to 15 ml tubes, lysed in 8 ml ice-cold NP-40 lysis buffer (1% NP-40, 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 15 mM MgCl2, 60 mM β-glycerolphosphate, 1 mM DTT, 0.1 mM NaVO4, 0.1 mM NaF, 15 mM p-nitrophenylphosphate, 1.8 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μg/ml

814
soybean trypsin inhibitor, 0.1 mM benzamidine), and filtered through 0.45 μm cellulose acetate membranes. Lyastes were then precluorated for 30 min with 1 ml packed Sepharose CL-4B (Pharmacia). HA1-CAN was immunoprecipitated by rotating the cleared lyses twice for 1 h with 0.4 mg of monoclonal antibody 2CAS (BAbCo, Newport, CA) covalently linked to 0.2 ml packed CNBr-activated Sepharose CL-4B beads (Pharmacia). The beads were washed four times with 8 ml NP-40 lysis buffer and then once with PBS. Proteins were eluted from the Sepharose beads by subsequent butylhexane elutions with 0.4, 0.4 and 0.2 ml 0.5% SDS, and vacuum concentrated (Speed-Vac) to 250 μl. Proteins in the eluates were subsequently precipitated with 5 volumes acetone at room temperature and pelleted at 18 000 × g for 10 min. The pellets were suspended in 25 μl solubilization buffer (10% SDS, 100 mM MgCl₂, 50 mM Tris-HCl pH 6.8, 0.1% bromophenol blue, 10% glycerol, 50 mM diithiothreitol) by vortexing and heating to 90°C. 50 μl of this fraction, which represents proteins purified from 4 g of TTD2 cells, were loaded in 7 mm wide slots and separated on a 0.75 mm 6% polyacrylamide gel. Mock samples containing solubilization buffer alone were run in adjacent lanes to prevent the protein bands from running off. After electrophoresis, proteins were stained with Coomassie brilliant blue R250 (Hoechst, Hercules, CA), and excised from the gel. The protein in the gel slices was quantitated by laser densitometry mass spectrometry (Williams et al., 1996), In-gel tryptic digestion, reversed-phase HPLC and amino acid sequencing were subsequently performed as described (Williams and Stone, 1995).

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