The nucleoporin-like protein NLP1 (hCG1) promotes CRM1-dependent nuclear protein export

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Summary
Translocation of transport complexes across the nuclear envelope is mediated by nucleoporins, proteins of the nuclear pore complex that contain phenylalanine-glycine (FG) repeats as a characteristic binding motif for transport receptors. CRM1 (exportin 1), the major export receptor, forms trimeric complexes with RanGTP and proteins containing nuclear export sequences (NESs). We analyzed the role of the nucleoporin-like protein 1, NLP1 (also known as hCG1 and NUPL2) in CRM1-dependent nuclear transport. NLP1, which contains many FG repeats, localizes to the nuclear envelope and could also be mobile within the nucleus. It promotes the formation of complexes containing CRM1 and RanGTP, with or without NES-containing cargo proteins, that can be dissociated by RanBP1 and/or the cytoplasmic nucleoporin Nup214. The FG repeats of NLP1 do not play a major role in CRM1 binding. Overexpression of NLP1 promotes CRM1-dependent export of certain cargos, whereas its depletion by small interfering RNAs leads to reduced export rates. Thus, NLP1 functions as an accessory factor in CRM1-dependent nuclear protein export.

Key words: NLP1, hCG1, Nuclear export, CRM1, Nucleus, Nup214

Introduction
Exchange of molecules between the cytoplasm and the nucleus occurs through nuclear pores that are embedded in the nuclear envelope. The nuclear pore complex (NPC) is a giant protein assembly with many copies of approximately 30 individual nucleoporins (Nups) [for a review see Wente and Rout and references therein (Wente and Rout, 2010)]. Four transmembrane Nups that anchor the NPC in the double lipid bilayer of the nuclear envelope have been described in vertebrate cells (Chadrin et al., 2010; Gerace et al., 1982; Hallberg et al., 1993; Mansfeld et al., 2006; Stavru et al., 2006). Approximately 15 different structural Nups form subcomplexes that participate in the formation of characteristic NPC building blocks such as the nuclear basket and nuclear or cytoplasmic rings. Finally, approximately 10 Nups are rich in phenylalanine–glycine (FG) repeats, motifs that mediate the interaction of the NPC with soluble nuclear transport receptors. This interaction is the basis of all models of the mechanisms for the selective translocation of macromolecules across the NPC (for reviews, see Goldfarb, 2009; Terry and Wente, 2009; Waldé and Kehlenbach, 2010; Wente and Rout, 2010). The majority of the soluble factors belong to the superfamily of importin-β-like transport receptors. They are called importins or exportins, depending on the major direction of cargo transport, and are collectively also referred to as karyopherins (for a review, see Fried and Kutay, 2003). The prototype importin is importin-β itself, which, through the adaptor protein importin-α, interacts with proteins containing a classical nuclear localization signal (cNLS). The major exportin is exportin 1, better known as CRM1 (Fornerod et al., 1997a; Fukuda et al., 1997; Kehlenbach et al., 1998; Neville et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997). CRM1 interacts with proteins containing a nuclear export sequence (NES) and mediates transport of hundreds of different proteins and certain RNAs out of the nucleus (for a review, see Hutten and Kehlenbach, 2007). The first CRM1-dependent NESs were identified in the HIV-1 Rev protein, which serves as an adaptor for nuclear export of unspliced viral RNAs (Fischer et al., 1995) and in the inhibitor of the catalytic subunit of cAMP-dependent protein kinase, PKI (Wen et al., 1995). Recently, the crystal structure of CRM1 in a complex with export cargos was determined (Dong et al., 2009; Monecke et al., 2009). An important component of export complexes is the small GTP-binding protein Ran in its GTP-bound form, which binds cooperatively to CRM1, together with export cargos (Fornerod et al., 1997a). Similar to nuclear import complexes, CRM1-containing export complexes can interact with FG Nups. Among these is Nup214, a nucleoporin that localizes to the cytoplasmic side of the NPC and that is required for efficient export of some, but not all, CRM1 cargos (Bernad et al., 2006; Bernad et al., 2004; Hutten and Kehlenbach, 2006). More recently, Nup98 was described as a CRM1-interacting nucleoporin that promotes nuclear export (Oka et al., 2010).

In this study, we investigated the role of the ill-defined nucleoporin-like protein NLP1 (also known as NUPL2) in humans, which has a presumptive ortholog, Rev-interacting protein (Rip1p; also known as Nup42), in yeast. Rip1p was originally identified in a yeast two-hybrid screen, searching for yeast proteins that bind to the viral protein HIV-1 Rev (Stutz et al., 1995). Later, the human protein human candidate gene 1 (hCG1) was described (Van Laer et al., 1997). hCG1 has 55% amino acid sequence homology to Rip1p over the entire length of the proteins (423 aa in humans) and can substitute for Rip1p functions in yeast (Strahm et al., 1999). hCG1 was also identified as NLP1 (nucleoporin-like protein 1), again as a protein...
interacting with HIV-1 Rev in a two-hybrid screen (Farjot et al., 1999). A characteristic of the yeast and the human proteins is the abundance of FG motifs, which are also found in FG nucleoporins. Although the yeast protein Rip1p is not essential, it seems to be required for export of certain heat shock mRNAs (Saavedra et al., 1997; Stutz et al., 1997). Another study suggested a role of Rip1p in nuclear export of heat shock and non-heat shock mRNAs at elevated temperatures (Vainberg et al., 2000). Rip1p was found to also interact with CRM1 in two-hybrid assays (Neville et al., 1997), as well as in vitro using purified proteins (Floer and Blobel, 1999). A Rip1p-deletion strain did not exhibit a profound defect in CRM1-dependent nuclear protein export (Stade et al., 1997). Hence, the functional relevance of Rip1p–CRM1–RanGTP complexes (Floer and Blobel, 1999) remains unclear. The human protein NLP1 interacts with the mRNA export factor TAP (Katahira et al., 1999) and functions in nuclear export of Hsp70 mRNA (Kendirgi et al., 2005). Similar to the yeast protein, an interaction between NLP1 and CRM1 was detected in two-hybrid assays (Farjot et al., 1999). Nothing is known, however, about the function of NLP1 in nuclear export of protein cargos in mammalian cells. Here, we describe a supportive role of NLP1 in CRM1-dependent export.

**Results**

**CRM1 concentrations are rate-limiting for nuclear export of artificial reporter proteins**

The cellular concentration of nuclear import factors of the importin-β superfamily is rate-limiting for efficient transport of proteins into the nucleus (Yang and Musser, 2006). We investigated whether the concentration of CRM1, the major nuclear export factor, is also rate-limiting, and analyzed the subcellular localization of nuclear shuttling proteins in control HeLa cells and in cells overexpressing CRM1. As a reporter protein, we used an artificial shuttling protein, a double GFP containing an N-terminal NES and a C-terminal cNLS (NES–GFP<sub>2</sub>–cNLS). This protein localized predominantly in the nucleus in ≤70% of transfected control cells (Fig. 1A,B). Strikingly, co-transfection of hemagglutinin-tagged CRM1 (CRM1–HA) resulted in a clear shift of the reporter protein towards the cytoplasm, suggesting that nuclear export of overexpressed NES–GFP<sub>2</sub>–cNLS is limited by the cellular CRM1 concentration. In the presence of leptomycin B (LMB), a selective CRM1 inhibitor (Wolff et al., 1997), NES–GFP<sub>2</sub>–cNLS, is localized entirely in the nucleus in control cells and in cells overexpressing CRM1–HA, indicating that nuclear export of the reporter protein indeed involves CRM1 as an export factor and also that its nuclear import was not inhibited under our experimental conditions. Very similar observations were made with another shuttling protein, the negative cofactor 2 β (NC2β; see supplementary material Fig. S1A,B). NC2β employs importin-α and/or -β and importin 13 as import- and CRM1 as export receptors, respectively (Kahle et al., 2009). Notably, under conditions of overexpression of CRM1 substrates, the cytoplasmic localization of the endogenous CRM1 cargo RanBP1 was not compromised, suggesting that the CRM1 system was not completely overloaded (supplementary material Fig. S1C). Together, these results demonstrate that in HeLa cells, CRM1 concentrations are not saturating for export of overexpressed nucleocytoplasmic shuttling proteins. We next analyzed the function of NLP1, a putative CRM1-binding protein, as a potential accessory factor that might stimulate nuclear protein export under certain conditions.

**NLP1 interacts with CRM1 in a RanGTP- and export cargo-dependent manner**

The yeast ortholog of NLP1, Rip1p/Nup42p, forms trimeric complexes with CRM1 and RanGTP. Export cargos such as HIV-1-Rev, however, appeared to be excluded from such complexes (Floer and Blobel, 1999). In yeast two-hybrid assays, human NLP1 has previously been described to interact with CRM1 (Farjot et al., 1999). A direct binding or a functional role in nuclear export, however, has not been reported. We therefore set out to analyze the binding of NLP1 to CRM1 using recombinant proteins, and to investigate the role of this protein in CRM1-dependent export. First, we expressed NLP1 fused to the maltose binding protein (MBP) and immobilized it on beads. CRM1 and RanQ69L, a Ran mutant that is insensitive to the GTPase-activating protein RanGAP (Klebe et al., 1995), loaded with GTP were then added in combination with an NES peptide or with GST–snurportin 1 (GST–SPN1) as a CRM1-dependent export cargo (Dong et al., 2009; Monecke et al., 2009; Paraskeva et al., 1997). Very little binding of CRM1 to NLP1 was observed in the absence of RanQ69L–GTP, whether or not an export cargo was present in the reaction (Fig. 2A, lanes 1, 2, 5, 6). Addition of RanQ69L–GTP alone resulted in detectable levels of CRM1 binding to NLP1 (lanes 3, 7). Including either GST–SPN1 (lane 4) or the NES–peptide (lane 8) in the reaction further increased CRM1 binding to MBP–NLP1, suggesting the formation of tetrameric complexes. Binding of GST–SPN1 was only detected when RanGTP was present in the reaction (compare lanes 2 and 4). Importantly, CRM1 and RanQ69L–GTP bound to immobilized MBP–NLP1 but not to MBP alone when added to the reaction together with an export cargo (lanes 9 and 10), demonstrating the specificity of the interaction. In a complementary experiment, we immobilized GST–Ran–GTP and analyzed binding of CRM1, MBP–NLP1 and SPN1. Again, the strongest signals were observed when CRM1 was added together with MBP–NLP1 and His–SPN1 (Fig. 2B, lane 6). Less CRM1 was detected when either His–SPN1 (lane 4) or MBP–NLP1 (lane 5) was omitted from the reaction. Only background binding was detected when the immobilized GST–Ran had been loaded with GDP (data not shown) or when unfused GST had

![Fig. 1. CRM1 concentrations are rate-limiting for nuclear protein export](image_url)
been immobilized (Fig. 2B, lane 8). To analyze the interaction of CRM1–NLP1 complexes with RanGTP and RanGDP in a more quantitative manner, we performed an ELISA-type binding assay. GST–Ran loaded with either GDP or GTP was bound to microtiter plate wells and incubated with MBP–NLP1 and increasing concentrations of CRM1. Bound NLP1 was then detected with a specific anti-NLP1 antibody. As shown in Fig. 2C, only background levels of NLP1 binding to immobilized RanGDP were observed, even at the highest CRM1 concentration. With immobilized RanGTP, by contrast, increasing CRM1 concentrations resulted in increased binding of NLP1. Especially at lower CRM1 concentrations, the addition of His–SPN1 to the reaction further promoted binding of NLP1 (together with CRM1) to RanGTP. With or without SPN1, half-maximal binding was observed at CRM1 concentrations in the low nanomolar range. These results show that NLP1 can form trimeric complexes with RanGTP and the export receptor CRM1. CRM1 cargos such as SPN1 or an NES peptide can join these complexes, suggesting that NLP1 functions in CRM1-mediated nuclear export rather than in re-import of CRM1 back into the nucleus.

We previously showed that wild-type RanGTP in a complex with CRM1 and the nucleoporin Nup214 is resistant to RanGAP-promoted GTP hydrolysis (Hutten and Kehlenbach, 2006). Likewise, a ternary complex of yeast CRM1, RanGTP and Rip1p/Nup42p was protected against RanGAP (Floer and Blobel, 1999). In RanGAP assays, a C-terminal fragment of Nup214 strongly reduced the ability of RanGAP to promote GTP hydrolysis on Ran in the presence of CRM1 (Fig. 3A), confirming our previous results (Hutten and Kehlenbach, 2006). Similarly, the addition of NLP1 to the reaction inhibited GTP hydrolysis. By contrast, Nup88, a nucleoporin that does not interact with CRM1, was not active in this assay, as shown before (Hutten and Kehlenbach, 2006). This result supports the notion that trimeric complexes containing RanGTP, CRM1 and NLP1 can form. In a late step of transport, such trimeric complexes (and of course tetrameric ones containing an export cargo) have to dissociate. The RanGTP-binding protein RanBP1 has previously been implicated in terminal steps of nuclear export (Kehlenbach et al., 1999). We therefore tested the ability of RanBP1 to disassemble NLP1-containing complexes. As before, tetrameric complexes containing immobilized MBP–NLP1, CRM1, RanQ69L–GTP and an NES-peptide were assembled. After the reaction, RanBP1 was added, and binding of CRM1 and Ran to NLP1 was analyzed. Clearly, RanBP1 dissociated the pre-assembled complexes because the levels of bound CRM1 and Ran were strongly reduced (Fig. 3B). Very similar observations were made with SPN1 as a CRM1 cargo (data not shown). Together, our results demonstrate the ability of NLP1 to interact with CRM1 and RanGTP, with or without export cargos, suggesting a role in nuclear protein export. Such complexes are expected to be stable until they are dissociated by the concerted action of cytoplasmic RanBP1 and RanGAP.

Nup214 is a well-characterized binding partner of CRM1 and is involved in nuclear export of a subset of proteins (Bernad et al., 2006; Fornerod et al., 1997a; Hutten and Kehlenbach, 2006). According to our previous results, it functions at a late stage of nuclear export, i.e. before disassembly of the RanBP1-mediated export complex (Hutten and Kehlenbach, 2006; Kehlenbach et al., 1999). We therefore asked whether NLP1 and Nup214 interact with CRM1 in a similar manner. CRM1–NLP1 complexes were assembled on immobilized wild-type GST–Ran, and increasing concentrations of an FG-rich C-terminal fragment of Nup214 that is known to interact with CRM1 (Fornerod et al., 1996) were added. Clearly, the Nup214 fragment prevented NLP1 from binding to the GST–Ran-CRM1 complex.
(Fig. 3C), suggesting that binding of the two proteins to CRM1 is mutually exclusive. Furthermore, the Nup214 fragment could dissociate a pre-assembled NLP1–CRM1–RanGTP complex (data not shown). Thus, nuclear export complexes might sequentially bind to NLP1 and Nup214 during transport, before complex disassembly at the cytoplasmic site of the NPC by RanBP1.

**FG repeats are not crucial for the NLP1–CRM1 interaction**

CRM1 has been shown to interact with a number of FG Nups (Neville et al., 1997). For Nup214, the FG-repeat-containing C-terminal part of the protein is required for high-affinity interaction with CRM1 (Fornerod et al., 1997b). We therefore analyzed the region(s) in NLP1 that are required for CRM1 binding. Various fragments and deletion mutants of NLP1 were expressed as either MBP or GST fusions, depending on the solubility of the proteins. The proteins were immobilized on beads and analyzed for CRM1 binding as described above.

Fig. 4A,B summarizes our results. The details of the binding experiments are shown in the supplementary material Fig. S2. Surprisingly, the FG repeats of NLP1 do not seem to play a major role in CRM1 binding. C-terminal fragments [amino acids (aa) 165–423 and 205–423], which contain the majority of the FG repeats, showed weak binding or no binding at all. An N-terminal fragment containing only two FG repeats (aa 1–204), by contrast, clearly interacted with CRM1 in an SPN1- and RanGTP-dependent manner. The putative coiled-coil region of NLP1 (aa 165–204) seems to contribute to CRM1-binding (compare the full-length protein, aa 1–423 and fragment 1–423Δ165–204, fragments 1–204 and 1–165 and fragments 165–423 and 205–423). In the deletion mutant lacking the coiled-coil region, a mutation of two FG repeats in the N-terminal part of NLP1 did not lead to reduced CRM1 binding. Together, our biochemical analysis shows that NLP1 can engage in interactions that are relevant for CRM1-dependent nuclear export. Interestingly, the FG repeats, the classic binding motif for nucleoporin–transport receptor interactions, do not seem to be absolutely required for CRM1 binding. We therefore generated an NLP1 mutant in which all phenylalanine residues within FG motifs were replaced with serine residues (NLP1FG-less). Clearly, this mutant interacted with CRM1 in a Ran–GTP- and cargo-dependent manner, albeit to a lesser extent than the wild-type protein (Fig. 4C).

**Fig. 3. RanGAP-resistant NLP1–CRM1–RanGTP complexes are dissociated by RanBP1 and Nup214.** (A) RanGAP assay. Increasing concentrations of MBP–NLP1 (full-length) or fragments of Nup214 or Nup88 were incubated with CRM1 and [γ-32P]RanGTP. GTP hydrolysis was initiated by the addition of RanGAP. (B) MBP–NLP1 was immobilized on beads and incubated with CRM1, RanQ69L–GTP and an NES peptide. After complex formation, RanBP1 was added for 60 minutes. (C) GST–Ran, loaded with GTP, was immobilized on beads and incubated with CRM1, MBP–NLP1 (5 μg each), an NES peptide and increasing amounts (2.5–10 μg) of MBP–Nup214 (aa 1859–2090). Bound proteins were analyzed by SDS-PAGE and Coomassie Blue staining (B,C).

**Fig. 4. FG repeats are not crucial for NLP1–CRM1 complexes.** (A) MBP- or GST-tagged fragments of NLP1. Orange box, zinc finger region; yellow box (C–C), putative coiled-coil region. FG motifs are depicted as vertical bars. Binding to CRM1 (as analyzed in B) was qualitatively assessed (+++, strong; ++, intermediate; +, weak; –, no binding). (B) NLP1 fragments were immobilized on beads and incubated with CRM1 in the presence or absence of His–SPN1 and RanQ69L–GTP, as indicated. See supplementary material Fig. S2 for entire gels. (C) Full-length NLP1FG-less was fused to MBP, immobilized as in B and incubated with CRM1 in the presence or absence of GST–SPN1 and RanQ69L–GTP, as indicated. Binding of CRM1 was analyzed by SDS-PAGE and Coomassie Blue staining (B,C).
NLP1 is found at the nuclear envelope and could be mobile within the nucleoplasm

In a proteomic analysis, mammalian NLP1 was suggested to be a component of the NPC, occurring at a copy number of 16 (Cronshaw et al., 2002). In NLP1-overexpressing HeLa cells, the protein was originally detected in the nucleus, being excluded from nucleoli (Farjot et al., 1999). A myc-tagged version of NLP1 also localized to the nuclear envelope in transfected cells (Le Rouzic et al., 2002). However, a thorough analysis of the subcellular and subnuclear localization of the endogenous protein has not been performed. We therefore raised antibodies against NLP1 in rabbits and also tested two anti-NLP1 antibodies raised in guinea pigs (data not shown). None of these antibodies yielded specific and reliable signals in indirect immunofluorescence assays, although they specifically recognized NLP1 in western blotting. We therefore resorted to a biochemical fractionation approach and also re-investigated the localization of NLP1 using various tagged proteins in overexpressing cells. As shown in Fig. 5A, endogenous NLP1 largely co-fractionated with nucleoporins, as detected with the anti-FG-Nup antibody mAb414. Only small amounts of NLP1 could be detected in the cytosolic fraction. When we expressed GFP–NLP1, RFP–NLP1, NLP1–RFP, HA–NLP1 or NLP1–HA, low expressing cells showed a clear signal at the nuclear envelope (Fig. 5B, arrows). Many cells also showed a clear nuclear staining, excluding the nucleoli (Fig. 5B and data not shown). In highly overexpressing cells, NLP1 was also detectable in the cytoplasm. In control cells, potential binding sites for NLP1 in the NPC are probably occupied by the endogenous protein. We therefore expressed GFP–NLP1 in cells where the endogenous protein had been depleted by specific siRNAs. The localization of GFP–NLP1 in such cells was very similar to that observed in control cells, with some cells exhibiting a weak signal for GFP–NLP1 at the nuclear rim and others a pan-nuclear signal (Fig. 5B, right panel). We next investigated whether nucleoplasmic GFP–NLP1 is a mobile protein by performing fluorescence recovery after photobleaching (FRAP) assays. As an example of a mobile protein, we expressed GFP–GST–cNLS, which is efficiently transported into the nucleus. A small nuclear region of GFP–GST–cNLS. This reduced mobility could be explained, for example, by transient interactions of NLP1 with nucleic acids. Together, our results suggest that NLP1 interacts with the nuclear envelope, in accordance with previous observations (Farjot et al., 1999; Le Rouzic et al., 2002). Depending on the expression level, it might also be found in the nuclear interior, similar to many other nucleoporins.

NLP1 promotes CRM1-dependent nuclear export

In light of our initial results showing that the concentration of CRM1 is rate-limiting for nuclear export (Fig. 1), we next expressed tagged versions of NLP1 in HeLa cells and analyzed their effects on nuclear transport. In cells that had been co-transfected with an empty HA vector, our artificial reporter protein NES–GFP2–cNLS localized mainly to the nucleus (Fig. 6A,B), similar to our results shown in Fig. 1. When cells were co-transfected with a construct coding for NLP1–HA, however, the percentage of cells showing a clear nuclear localization of NES–GFP2–cNLS dropped to ~50%. Such changes in the subcellular localization of NES–GFP2–cNLS could result from stimulated export or inhibited import. To distinguish between these two possibilities, we incubated transfected cells in the presence of LMB. This treatment resulted in a strong nuclear accumulation of NES–GFP2–cNLS, and also occurred in cells expressing NLP1–HA (Fig. 6B). Hence, overexpression of NLP1–HA did not inhibit nuclear import of NES–GFP2–cNLS but rather stimulated its nuclear export. Similar observations were made with NC2β–GFP2 as a reporter protein and N- or C-terminally RFP-tagged NLP1. Again, co-expression of NLP1 fusion proteins resulted in a clear shift of NC2β–GFP2 towards the cytoplasm, compared with cells expressing unfused RFP (Fig. 6C,D). As above, treatment of cells

![Fig. 5. NLP1 is a mobile nuclear protein.](image-url)
with LMB led to a clear nuclear accumulation of NC2β–GFP₂ under all conditions, indicating stimulated nuclear export in cells expressing RFP–NLP1 or NLP1–RFP. Next, we analyzed the kinetics of nuclear export in control cells and in cells overexpressing NLP1. To this end, we performed fluorescence loss in photobleaching (FLIP) assays in cells co-expressing NC2β–GFP₂ and either RFP or RFP–NLP1. A cytoplasmic region in RFP-positive cells was constantly bleached and the loss of GFP fluorescence in the nuclear compartment was measured as an indicator of the efficiency of nuclear export of NC2β–GFP₂. As shown in Fig. 6E, cells co-expressing RFP–NLP1 exhibited faster export kinetics than cells co-expressing RFP.

NLP1 is a nuclear-envelope-associated protein that could potentially also shuttle between the nuclear envelope and the nuclear interior. Given the stimulatory effect of NLP1 on nuclear export in vivo, we argued that recombinant NLP1 might also promote CRM1-dependent nuclear export in permeabilized cells. HeLa cells that stably expressed GFP–NFAT [nuclear factor of activated T cells (Kehlenbach et al., 1998)] were permeabilized with digitonin and subjected to nuclear export reactions in the presence of Ran and CRM1, with or without additional NLP1. Clearly, NLP1 promoted nuclear export of GFP–NFAT (supplementary material Fig. S3). This stimulation of export was specific, as the loss of nuclear fluorescence in the presence of NLP1 required ATP. Furthermore, nuclear export under all conditions was inhibited by wheat germ agglutinin, a general inhibitor of nuclear transport (data not shown), demonstrating that the permeability barrier of the NPC was not compromised during the reaction.

Our results, described so far, suggest that NLP1 stimulates CRM1-dependent nuclear export under conditions where the export receptor itself is rate-limiting. This limitation might result from low concentrations of CRM1 and/or from comparatively low affinities of export cargos to the receptor.

Depletion of NLP1 inhibits CRM1-dependent nuclear export

In in vitro reactions using digitonin-permeabilized cells, CRM1 and Ran were the only exogenous factors that were required for efficient nuclear export (Kehlenbach et al., 1998). Under these conditions, however, NLP1 is still present in nuclei of permeabilized cells (see Fig. 5A). To specifically address the question of whether NLP1 is required for CRM1-dependent export, we performed siRNA experiments, reducing the cellular concentration of the protein by RNA interference. In a similar setup, we previously showed that depletion of nucleoporin Nup214, but not Nup358 reduced CRM1-dependent nuclear protein export (Hutten and Kehlenbach, 2006). As judged by western blotting, two different siRNAs against NLP1 resulted in a clear reduction of the NLP1 concentration, although traces of the protein were still detectable (Fig. 7A and data not shown). Other proteins that are involved in various steps of nucleocytoplasmic transport were not affected by the siRNA treatment (Fig. 7A). We first investigated nuclear export of our reporter protein GFP–NFAT (Hutten and Kehlenbach, 2006). Cells were treated with ionomycin to induce nuclear import of the protein. Control cells and siRNA-treated cells showed the same partial nuclear import of GFP–NFAT after 1 minute and complete nuclear localization of the reporter protein after 2
Discussion

Limiting CRM1 concentrations: a means to regulate transport?

CRM1 is an export receptor that interacts with hundreds or even thousands of cargo proteins. Strikingly, CRM1 uses the same hydrophobic binding pocket for interaction with different cargos, forcing NES peptide sequences to adapt their conformation to the rather rigid binding-site on CRM1 (Güttinger et al., 2010). Depending on the spacing of key hydrophobic residues, the affinities of NES-containing cargos for their cognate receptor CRM1 can vary dramatically, with low-affinity cargos prevailing (Cook et al., 2007; Kutay and Güttinger, 2005). Functionally, weak affinities seem to be important for efficient disassembly of export complexes on the cytoplasmic side of the NPC (Engelsma et al., 2004). Furthermore, they prevent cargos from binding to CRM1 in the cytoplasm in the absence of RanGTP (Kutay and Güttinger, 2005). The consequences of different affinities on the subcellular localization of individual nucleocytoplasmic shuttling proteins has not been investigated so far. Our results now show that the cellular CRM1 concentrations are rate limiting for nuclear export in HeLa cells, at least under conditions of overexpression of cargo proteins. Elevated CRM1 levels have been observed in many tumor cells including cervical cancers (Noske et al., 2008; van der Watt et al., 2009) and could also be effective in our HeLa cells. Hence, export of a subset of endogenous proteins in primary cells could well be limited under certain conditions by the available pool of the export receptor, allowing the cells to regulate export simply by adjusting the CRM1 concentration. Under conditions where many NES-containing proteins compete for binding sites on CRM1, the formation of export complexes containing low-affinity CRM1 substrates could also be promoted by accessory factors. One such factor is the Ran-binding protein RanBP3, which enhances the affinity of CRM1 for cargo proteins and for RanGTP. As a consequence, RanBP3 stimulates CRM1-dependent export in permeabilized cells (Engelmeier et al., 2001; Lindsay et al., 2001). Another cofactor in CRM1-dependent export is the nucleoporin Nup98, which also interacts with the export receptor in a RanGTP-dependent manner (Oka et al., 2010). Here, the authors used antibody-injection experiments and suggested a role of Nup98 in export of overexpressed transport cargos in living cells.
In our study, we analyzed the role of a nucleoporin-like protein, NLP1, in CRM1-mediated export. NLP1 had previously been implicated in nuclear export of certain mRNAs (Katahira et al., 1999; Kendirgi et al., 2005). Although it had been shown to interact with CRM1 in two-hybrid assays, its function in protein export in mammalian cells has remained unclear. We now show that NLP1 promotes CRM1-dependent export of certain cargos in vitro and in vivo. Together with other accessory factors, NLP1 might contribute to the control of nuclear export of distinct cargo proteins.

NLP1 promotes CRM1-dependent export

NLP1 was originally so named because of the characteristic FG repeats, as they also occur in certain nucleoporins. Indeed, the protein was identified as a component of the NPC in a proteomic analysis (Cronshaw et al., 2002). In one study, the yeast homologue Rip1p/Nup42p has been found to reside in the nucleus and at the nuclear and the cytoplasmic face of the NPC (Strahm et al., 1999) and in another study exclusively on the cytoplasmic side of the NPC (Rout et al., 2000). Our results now suggest that mammalian NLP1 is associated with the nuclear envelope and could also be mobile within the nucleus, similar to Nup98. The levels of intranuclear NLP1 could well depend on the expression level of the NLP1 gene. To unequivocally demonstrate the existence of an intranuclear pool of NLP1 and further analyze its significance, specific antibodies that are suitable for immunofluorescence or immunoelectron microscopy will be required.

Overexpression as well as siRNA depletion experiments point to a role of NLP1 in CRM1-mediated nuclear protein export. The results of the overexpression experiments can be easily explained if we assume that a nuclear pool of NLP1 does exist under physiological conditions and could thus support complex formation as discussed below. If a nuclear pool only resulted from overexpression of the protein, we would have to assume that the levels of endogenous NLP1 at the nuclear envelope are not saturated in all cells. Upon overexpression, additional binding sites at the nuclear envelope and/or nuclear pore are then occupied by NLP1, resulting in stimulation of CRM1-dependent export. However, the siRNA depletion experiments in which we observed a small reduction of CRM1-dependent transport, suggest that NLP1 is not absolutely required for nuclear export. Instead, it functions as an accessory factor, e.g. for proteins that on their own have a low affinity for the export receptor. In cells that do not overexpress transport receptors as many cancer cells do (Noske et al., 2008; van der Watt et al., 2009), limiting CRM1 concentrations might prevail. It will be interesting to investigate the functional relevance of NLP1 in such cells in the future.

In binding assays, we could demonstrate that: (1) NES cargos as well as RanGTP enhanced binding of CRM1 to NLP1; (2) NLP1 enhanced binding of cargo to CRM1–RanGTP complexes; and (3) CRM1 (indirectly) enhanced binding of NLP1 to RanGTP. These results suggest that distinct trimeric and tetrameric export complexes can assemble in the nucleus. Strikingly, the FG repeats of NLP1 do not play a major role in CRM1 binding, although an FG-rich region of Nup214 seems to compete for the same binding site on CRM1. It will be interesting to compare the interaction mode of CRM1 with the two proteins in detail.

High-affinity NES cargos might form trimeric export complexes with RanGTP and CRM1 and be exported as such. Alternatively, a trimeric ‘pre-export complex’, containing CRM1, RanGTP and NLP1 might initially form in the nucleus or on the nuclear side of the NPC and accommodate a fourth component, a high- or low-affinity NES cargo. In such trimeric and tetrameric complexes, Ran is resistant to RanGAP-induced GTP hydrolysis. RanGAP, which promotes GTP hydrolysis by more than 1000-fold (Bischoff et al., 1994) is restricted to the cytoplasm but could occasionally enter the nucleus, leading to deleterious effects on the Ran gradient. In that sense, NLP1 would help to protect nuclear Ran from GTP hydrolysis. Furthermore, it would compensate for low affinities of NESs, which on their own are not sufficient for tight CRM1 binding, at least not under conditions where high-affinity cargos are competing for the same binding site. How could such a mechanism facilitate export of low-affinity cargos, when also high-affinity CRM1 substrates, such as SPN1, would profit from enhanced export complex formation? In the simplest scenario, certain NES cargos could directly interact with NLP1, prior to export complex formation, resulting in a stabilization of the tetrameric (cargo–NLP1–CRM1–RanGTP) complex. Indeed, our initial results suggest that the HIV-1 Rev protein, a CRM1 cargo of comparatively low affinity (Paraskeva et al., 1999), can directly interact with NLP1, independent of CRM1 or RanGTP (data not shown). In such a complex, the NES of the cargo protein might be exposed upon NLP1 binding, promoting subsequent CRM1 interaction. Alternatively, NLP1 could level the differences in affinities as observed in direct cargo–CRM1 interactions. In that case, increasing the cellular concentration of NLP1 (or regulating its activity by post-translational modifications) would have a more profound effect on low-affinity CRM1 cargos than on high-affinity substrates. A similar observation was made for RanBP3, which increased the binding of a GST–NES substrate to CRM1 while decreasing binding of SPN1 (Englmeier et al., 2001).

After complex formation in the nucleus, the FG-rich C-terminal region of NLP1, which is not required for CRM1 binding, could facilitate translocation of the export complex through the transport channel of the NPC. Subsequently, disassembly of export complexes is the terminal step of transport on the cytoplasmic side of the NPC. In the simplest model, this is accomplished by the concerted action of RanBP1 or the RanGTP binding domains of the cytoplasmic nucleoporin Nup358 (RanBP2) and the GTPase-activating protein RanGAP (Kehlenbach et al., 1999). For export complexes containing NLP1 as an integral component, an additional factor could come into play. Nup214, a bona fide FG nucleoporin that also localizes to the cytoplasmic side of the NPC, binds to CRM1–RanGTP–NES complexes with high affinity (Hutten and Kehlenbach, 2006). Binding of NLP1 and Nup214 to such complexes is mutually exclusive (Fig. 3C), and Nup214 could replace NLP1 in the complex. It will be interesting to compare the interaction of CRM1 with the two proteins at the structural level, because FG repeats appear to be important for Nup214 binding but not for NLP1 binding. The export complex, now tethered to Nup214, would be well positioned for disassembly by RanBP1 and RanGAP, a large portion of which stably associates with Nup358 (Mahajan et al., 1997; Matunis et al., 1996). Nup358 also contains four Ran-binding domains that are functionally equivalent to that of RanBP1. Hence, soluble cytoplasmic factors would be dispensable for export complex dissociation, in line with previous observations (Kehlenbach et al., 1999). Interestingly, depletion of NLP1 reduced CRM1-dependent export of nuclear proteins.
export of NCβ and NFAT, two proteins that are also affected by the depletion of Nup214 (Stephanie Roloff and R.H.K., unpublished observations) (Hutten and Kehlenbach, 2006). Together, our data suggest a new function for NLP1 in promoting CRM1-dependent nuclear export of a subset of proteins.

Materials and Methods

Cell culture and transfections

HeLa P4 cells (Charneau et al., 1994) and HeLa NFAT cells (Hutten and Kehlenbach, 2006) were grown in DMEM (Gibco) containing 4.5 g/l glucose, 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were transfected using the calcium phosphate method (Ausubel et al., 1994) or Polyfect transfection reagent (Qiagen) according to the instructions of the manufacturer and cultured for 48 hours.

Plasmids

 Constructs coding for CRM1–HA (Hilliard et al., 2010), NC2 (Hutten and Kehlenbach, 2006) were grown in DMEM (Gibco) containing 4.5 g/l glucose, 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were transfected using the calcium phosphate method (Ausubel et al., 1994) or Polyfect transfection reagent (Qiagen) according to the instructions of the manufacturer and cultured for 48 hours.

Protein expression and purification

MBP-NLP1 and GST-NLP1 fusions were expressed in Escherichia coli BL21(DE3) cells upon induction with 500 µM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 hours at 24°C. Cells were lysed in 2 mM EDTA, 1% Triton X-100, 5 mM diithiothreitol (DTT), 1 µg/ml each of aprotinin, leupeptin and pepstatin, and centrifuged for 30 minutes at 100,000 g. After diluting the supernatant 1:5 with PBS, 10 mM MgCl₂, 5 mM DTT, 1 mM NaCl, 1 mM DTT and 1 µg/ml each of aprotinin, leupeptin and pepstatin, proteins were purified by affinity chromatography using amylose–resin beads (New England Biolabs) for MBP-tagged fragments or glutathione–Sepharose (GE Healthcare) for GST fusion proteins. After several washing steps, proteins were eluted with either 20 mM glutathione or 10 mM maltose in 10 mM Heps pH 8.0, 5% glycerol, 150 mM NaCl, 1 mM DTT and 1 µg/ml of protease inhibitors. His-NLP1 (1–204) was expressed as described for MBP-NLP1. For purification, a bacterial pellet was dissolved in 50 mM Tris pH 8.0, 2% Triton X-100, 2 mM DTT and 1 µg/ml each of aprotinin, leupeptin and pepstatin. After centrifugation for 20 minutes at 100,000 g, the pellet was resuspended in 50 mM Tris pH 8.0, 4 M urea, 1 mM DTT and protease inhibitors. After centrifugation as above, the supernatant was added to Ni-NTA agarose beads (Qiagen) and incubated for 1 hour. After several washing steps, the protein was eluted with binding buffer containing 300 mM imidazole and dialyzed against PBS. Precipitated protein was used for antibody production. GST–Ram and His-SNPN1 were expressed in BL21(DE3) upon induction with 500 µM isopropyl-β-D-thiogalactopyranoside and purified according to standard protocols. GST–SNPN1 (Strasser et al., 2004), RanBP1 (Kehlenbach et al., 2001), RanGAP (Mahajan et al., 1997), CRM1–His (Guan et al., 2000), Ran and RanQ69L (Melchior et al., 1995), MBP–Nup88 (aa 500-741) and MBP–Nup214 (aa1859–2090) (Hutten and Kehlenbach, 2006) were purified as described before. His-Nup214 was expressed in BL21(DE3) and purified as described above for MBP-NLP1 using Ni-NTA agarose beads and 300 mM imidazole for protein elution. All proteins were dialyzed against transport buffer (TPB; 20 mM Hepes-KOH, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT and 1 µg/ml each of aprotinin, leupeptin, pepstatin), frozen in liquid nitrogen and stored at −80°C. Ran was loaded with GTP, GDP or [γ-32P]GTP as described previously (Kehlenbach et al., 1999).

RNA interference

Cells were transfected with small interfering RNAs (siRNAs) from Ambion (NLP1 siRNA, 5′-agguaauanac gagcaggatt-3′, corresponding to nucleotides 120–138 and NLP1 siRNA5, 5′-gagguaauanac gagcaggatt-3′, corresponding to nucleotides 257–275) at a final concentration of 100 nM using Oligofectamine (Bioltegron), according to the instructions of the manufacturer. Control cells were treated with transfection reagent only. After 24 hours of siRNA treatment, cells were transfected with reporter protein constructs of interest and analyzed after 20–24 hours.

Antibodies

Antibodies against NLP1 were raised in rabbits by injecting His–NLP1 1–204 and affinity-purified using MBP–NLP1 coupled to CNBr beads. For the detection of HA-epitope-tagged proteins by immunofluorescence, a monoclonal mouse anti-HA antibody (16B12, Covance) was used. The rabbit anti-Nup214 antibody (Hutten and Kehlenbach, 2006) and the goat anti-RanGAP antibody (Hutten et al., 2008; Pichler et al., 2002) were described previously. The mouse anti-lamin A/C, anti-Ran, anti-RanBP1 and anti-RanBP3 antibodies were obtained from BD Bioscience, the mouse anti–α-tubulin antibody was obtained from Sigma. The anti-CRM1 antibody was raised in goat against a C-terminal decapetide as described before (Kehlenbach et al., 1998). The goat-anti Ubα2 antibody was a kind gift from Frauke Melchior (University of Heidelberg, Germany). For immunofluorescence, donkey anti-mouse Alexa Fluor 594 and donkey anti-rabbit Alexa Fluor 594 (1:1000; Molecular Probes) were used as secondary antibodies. For immunoblotting, HRP-coupled donkey anti-goat, donkey anti-mouse or donkey anti-rabbit IgG (1:5000; Dianova) were used as secondary antibodies.

Indirect immunofluorescence and microscopy

Immunofluorescence staining was performed as described before (Hutten and Kehlenbach, 2006). Cells were analyzed using a Zeiss Axioskop 2 microscope with a 100 × Plan-Neofluar 1.3 NA water-corrected objective and appropriate filter settings and processed using AxioVision Rel. 4.8 LE and Adobe Photoshop 6.0. For quantification of subcellular localization of reporter proteins, cells were grouped into two categories: N; predominant localization of proteins in the nucleus and C; predominant cytoplasmic localization. Quantification was performed from at least three independent experiments, counting more than 100 cells with similar expression levels. Statistical significance of the data was analyzed by a two-tailed, heteroscedastic Student’s t-test. P-values <0.05 were considered as statistically significant.

Live cell imaging

FRAP and FLIP analyses were performed at 37°C with a LSM 510 Meta confocal microscope (Zeiss) using a LCI Plan-Neofluor 63 × 1.3 NA water-corrected objective in a temperature-controlled chamber. Cells were grown on LabTec-chambers (Nunc) and transferred to CO₂-free medium (Invitrogen) before the analysis. The mobility of GFP–NLP1 was analyzed by FRAP. After 20 scans at low laser intensity (1.2% 488-nm laser transmission), an area of 10 × 30 pixels (13.35 × 4.15 µm) in the nucleus was bleached (100% transmission of the 488-nm laser, 40 iterations). Subsequently, scanning of the bleached area was continued at low laser intensity for 380 pictures (154 ms/second/picture). Data analysis was performed with LSM software (Zeiss) and Excel (Microsoft), normalizing the original fluorescence in the bleached area to one. FLIP analysis of nuclear export was performed essentially as described before (Hilliard et al., 2010).

Nuclear transport in HeLa-cells expressing GFP–NFAT

Nuclear import of GFP–NFAT (Hutten and Kehlenbach, 2006) was induced by the addition of 300 nM ionomycin (Sigma) and cells were incubated at 37°C for various periods of time, fixes with 4% formaldehyde and analyzed by fluorescence microscopy. Export of GFP–NFAT was analyzed as described previously (Hutten and Kehlenbach, 2006).

Binding studies

MBP or GST fusion proteins (5 µg) were immobilized on 20 µl amyllose–agarose or glutathione–agarose beads that had been preincubated with 10 mg/ml BSA in TPB. The beads were incubated with 5 µg of each of protein of interest or 10 µM NESP peptide [NS2 protein of minute virus of mice, CVD3KMkKGTlTlHIDTEK (Askjaer et al., 1999)] in 300 µl TPB containing 2 mM mg/ml BSA. After 75 minutes at 4°C, beads were washed three times with TPB. Bound proteins were eluted with SDS sample buffer and subjected to SDS-PAGE, followed by Coomassie Blue staining or western blotting.

ELISA

A 96-well plate (ImmuNo 96 MicroWell™ Solid Plates; Nunc) was coated overnight at 4°C with 300 ng GST–Ram per well loaded with GDP or GTP in 50 µl TPB. The wells were blocked with 3% BSA in TPB for 30 minutes at room temperature and 300 ng MBP–NLP1 and increasing concentrations of CRM1–His, with or without 300 ng His–SNPN1, were added. After incubation for 75 minutes at room temperature and several washing steps, bound NLP1 was detected using the rabbit anti-NLP1 antibody (1:5000 in TPB + 3% BSA) and HRP-conjugated donkey anti-rabbit IgG (1:2000 in TPB + 3% BSA) as secondary antibody. For the colorimetric reaction, the TMB substrate reagent set from Millipore was used. The
absorbance was measured at 420 nm using a plate reader (Applikon, Thermo Electron Corporation).

Cell fractionation
HeLa cells were trypsinized, washed with PBS and permeabilized with 0.01% digitonin in PBS with 1 μg/ml each of aprotinin, leupeptin and pepstatin. Nuclei were separated from cytosol by centrifugation at 1000 g for 5 minutes and washed twice with PBS. Fractions corresponding to 100,000 cells were analyzed by SDS-PAGE, followed by western blotting.

SDS-PAGE and western blotting
Proteins were analyzed by SDS-PAGE and western blotting using standard methods. The ECL system (Millipore) was used for visualization of proteins.

RanGAP assays
RanGAP assays were performed as described previously (Askjaer et al., 1999; Kahlenbach et al., 2001), with 16 nM RanGAP and increasing concentrations of MBP-NLP1, MBP-Nup214 (aa 1859–2090) or MBP–Nup88 (aa 500–741).

In situ hybridization
In situ hybridization was performed as described previously (Hutten and Kehlenbach, 2006).

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References


Fig. S1

A

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<td>[Image of green fluorescence]</td>
<td>[Image of red fluorescence]</td>
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</table>

B

![Bar graph showing percentage of cells with NC2β-GFP<sub>2</sub> under control and CRM1-HA conditions with and without LMB treatment.](image)

C

<table>
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<td>RanBP1</td>
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<tr>
<td>[Image of green fluorescence]</td>
<td>[Image of red fluorescence]</td>
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+LMB

Bar graph showing percentage of cells with NC2β-GFP<sub>2</sub> under control and CRM1-HA conditions with and without LMB treatment.
Fig.

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<th>GST-NLP 1-204</th>
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<tr>
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**Legend:**
- **CRM1**
- **His-SPN1** (*
- **BSA**
- **Ran**

**Diagram:**
- **MBP-NLP**
- **GST-NLP**
- **MBP**
- **GST**