The nuclear pore component Nup358 promotes transportin-dependent nuclear import

Saskia Hutten1,2, Sarah Wälde1, Christiane Spillner1, Joachim Hauber3 and Ralph H. Kehlenbach1,*

1Department of Biochemistry I, Faculty of Medicine, Georg-August-University of Göttingen, Humboldtallee 23, 37073, Göttingen, Germany
2Wellcome Trust Centre for Gene Regulation and Expression, MSI/WTB Complex, University of Dundee, Dundee DD1 5EH, UK
3Heinrich-Pette-Institute for Experimental Virology and Immunology, Martinistrasse 52, 20251 Hamburg, Germany

*Author for correspondence (e-mail: rkehrlen@gwdg.de)

Summary

Nup358 (also known as RanBP2), a component of the cytoplasmic filaments of the nuclear pore complex, has been implicated in various nucleocytoplasmic transport pathways. Here, we identify Nup358 as an important factor for transportin-mediated nuclear import. Depletion of Nup358 resulted in a strong inhibition of nuclear import of the human immunodeficiency virus type 1 (HIV-1) Rev protein. HIV-1 Rev is an RNA-binding protein that is required for CRM1 (also known as exportin 1)-dependent nuclear export of unspliced or partially spliced viral RNA. We show that transportin is the major nuclear import receptor for HIV-1 Rev in HeLa cells. Overexpression of transportin strongly promoted nuclear import of HIV-1 Rev in Nup358-depleted cells, indicating that the import receptor becomes rate-limiting under these conditions. Importantly, the import rate of other transportin-dependent proteins was also significantly reduced in Nup358-depleted cells. Our data therefore suggest a general role for Nup358 in transportin-mediated nuclear import.

Introduction

The transport of macromolecules across the nuclear membrane is a receptor-mediated, signal-dependent process. The transport machinery consists of a soluble phase, which includes the transported substrates, their transport receptors as well as regulatory proteins, and a stationary phase, the nuclear pore complex (NPC). Most of the transport receptors, also referred to as importins and exportins or, collectively, as karyopherins, belong to the importin-β superfamily (for a review, see Fried and Kutay, 2003). They interact with the small GTP-binding protein Ran, a regulatory cofactor in nucleocytoplasmic transport. In nuclear export, RanGTP is an integral component of the transport complex. Import receptors, by contrast, dissociate from their transport substrate upon interaction with RanGTP (Rexach and Blobel, 1995). A high nuclear concentration of RanGTP is maintained by the chromatin-associated nucleotide-exchange factor RCC1 (Bischoff and Ponstingl, 1991) and a dedicated nuclear-import factor for Ran, NTF2 (Ribbeck et al., 1998; Smith et al., 1998). Together, they promote the formation of export complexes and the disassembly of import complexes in the nucleus. In the cytoplasm, the concerted action of the GTPase-activating protein RanGAP together with the Ran-binding protein transportin results in the hydrolysis of RanGTP to RanGDP (for a review, see Fried and Kutay, 2003). RanGAP occurs either as a soluble, cytoplasmic protein or, upon modification with the small ubiquitin related modifier, SUMO, in an NPC-associated form (Mahajan et al., 1997; Matunis et al., 1996). Transport substrates contain characteristic sequences that mediate binding to transport receptors. Proteins bearing so-called leucine-rich nuclear-export sequences (NESs) are recognised by CRM1 (also known as exportin 1), the major export receptor for proteins (Fornerod et al., 1997; Fukuda et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997). Nuclear-localisation signals (NLSs), by contrast, mediate binding to importins. The best-characterised NLS is the ‘classical’ NLS (cNLS), a short stretch enriched in basic amino acids, which mediates import via the importin-α–importin-β heterodimer (for a review, see Fried and Kutay, 2003). In this case, importin-α serves as an adapter protein between cargo and the actual transport receptor importin-β. Importin-β has also been reported to bind directly to certain substrates, for example HIV-1 Rev, Fos or the parathyroid hormone-related protein (PTHrP) (Forwood et al., 2001; Henderson and Percipalle, 1997; Lam et al., 1999; Truant and Cullen, 1999), without the need for an adaptor protein. Another well-characterised import pathway depends on the importin-β-related transport receptor transportin. The transportin-dependent NLS was first described as a ~39-amino-acid region, also known as the M9 sequence, present in the heterogeneous ribonucleoprotein hnRNP A1 (Pollard et al., 1996; Siomi and Dreyfuss, 1995). Recently, a 19-amino-acid sequence, the M9 core, was shown to resemble a sufficient for transportin-mediated import (Iijima et al., 2006). Transportin is also known to be involved in nuclear import of a number of proteins lacking a typical M9-like sequence, such as the Fos protein (Arnold et al., 2006b). Additional importin-β-like transport receptors include importins 5, 7, 9 and 13 (Jäkel and Görlich, 1998; Mingot et al., 2001).

In addition to binding to their transport substrate and Ran, karyopherins interact with constituents of the NPC during the process of nucleocytoplasmic translocation. The NPC, a channel-forming complex of 125 MDA in vertebrate cells, is embedded between the inner and outer nuclear membrane (for reviews, see Fahrenkrog and Aebi, 2003; Tran and Wente, 2006). It consists of ~30 different proteins, so-called nucleoporins, about a third of which contain FG (phenylalanine, glycine) repeats. In various models, these FG repeats create a hydrophobic milieu for selective translocation of transport receptors, which can cross the NPC either...
The role of individual nucleoporins, however, is still controversial. Nucleoporins that localise asymmetrically to one side of the NPC could function as a docking station for transport complexes on their way into or out of the nucleus. Examples are the vertebrate nucleoporins Nup153 and Nup358, which localise to either the nuclear or to the cytoplasmic side of the NPC, respectively (Wu et al., 1995; Yokoyama et al., 1995). In yeast, however, asymmetric nucleoporins have been reported to be dispensable for nucleocytoplasmic transport (Strawn et al., 2004). Furthermore, in Xenopus oocytes, the cytoplasmic filaments containing Nup358 (also known as RanBP2) were reported to be dispensable for cNLS- and M9-mediated nuclear import in vitro (Walther et al., 2002). In Drosophila, however, Nup358 appears to be important for the import of several proteins in vivo (Forler et al., 2004; Sabri et al., 2007). Nup358 has been identified as the interaction partner for sumoylated RanGAP (Mahajan et al., 1997; Matunis et al., 1996). We previously showed that the Nup358-RanGAP complex is required for efficient importin-α/β-dependent import in vertebrate cells (Hutten et al., 2008).

The HIV-1 Rev protein is a shuttling protein that is required for the export of unspliced or partially spliced viral mRNAs out of the nucleus (for a review, see Pollard and Malim, 1998). It was one of the first proteins identified to contain a leucine-rich NES and can interact directly with importin-β (Henderson and Percipalle, 1997; Truant and Cullen, 1999). Recently, we demonstrated that additional import receptors, namely importin-5, importin-7 and transportin, bind to the same NLS and mediate the import of HIV-1 Rev in vitro (Arnold et al., 2006a). The identity of the endogenous Rev import receptor, however, remains to be elucidated.

In this study, we investigated the requirements for soluble and stationary components of the nucleocytoplasmic transport machinery for the nuclear import of HIV-1 Rev in living cells. Our data suggest that transportin is the major Rev import receptor in vivo, at least in HeLa cells. Furthermore, nuclear import of HIV-1 Rev strongly depends on the nucleoporin Nup358. Because nuclear import of other transportin-dependent substrates was also impaired in Nup358-depleted cells, we suggest a general role for Nup358 as a docking station for transportin-containing import complexes on their route into the nucleus.

**Results**

HIV-1 Rev is preferentially imported by transportin and importin-9 in vitro

Recently, we reported that importin-β, importin-5, importin-7 and transportin function as import receptors for HIV-1 Rev in vitro (Arnold et al., 2006a). In the previous study, we used an excess of import receptors for the import of GST-tagged Rev. To investigate whether HIV-1 Rev is imported preferentially by one of the transport receptors, in the present study we analysed its import in vitro with reduced concentrations of transport receptors using a His-YFP fusion of HIV-1 Rev as a reporter protein and also included importin-9 in our analysis. No or only little nuclear import of YFP-Rev was observed in the absence of import receptors (buffer) or in the presence of equimolar concentrations of importin-β, importin-13, importin-7 or importin-5 (Fig. 1A). By contrast, very efficient nuclear accumulation of Rev was obtained by the addition of equimolar concentrations of transportin and importin-9 in the presence of either wild-type Ran (RanWT), RanQ69L or WGA at 23°C. (C) GST-Rev was immobilised on beads and incubated with transportin or importin-9 in the absence or presence of RanQ69L-GTP. Bound proteins were analysed by SDS-PAGE followed by immunoblotting using anti-His antibodies. The input (inp.) corresponds to 10% of the import receptor used for the binding reaction. The faster-migrating band in the input lane for importin-9 is probably a degradation product that also interacts with GST-Rev in the absence but not in the presence of RanGTP. Scale bars: 10 μm.

**Fig. 1.** HIV-1 Rev is preferentially imported by transportin and importin-9 in vitro. (A) Digitonin-permeabilised cells were incubated with His-YFP-Rev as an import substrate and with equimolar concentrations of import receptors in the presence of 2 μM wild-type Ran at 4°C or 23°C, as indicated. trn, transportin. (B) Nuclear-import reactions were performed with reduced concentrations of either transportin or importin-9 (fourfold excess of His-YFP-Rev), as indicated, in the presence of either wild-type Ran (RanWT), RanQ69L or WGA at 23°C. (C) GST-Rev was immobilised on beads and incubated with transportin or importin-9 in the absence or presence of RanQ69L-GTP. Bound proteins were analysed by SDS-PAGE followed by immunoblotting using anti-His antibodies. The input (inp.) corresponds to 10% of the import receptor used for the binding reaction. The faster-migrating band in the input lane for importin-9 is probably a degradation product that also interacts with GST-Rev in the absence but not in the presence of RanGTP. Scale bars: 10 μm.
specific, as it could be inhibited by the addition of the Ran mutant RanQ69L (Fig. 1B). This Ran mutant does not hydrolyse its bound GTP (Klebe et al., 1995) and therefore dissociates functional importin-cargo complexes. To control the integrity of the nuclear envelope under our experimental conditions, we also included the lectin wheat-germ agglutinin (WGA) in the reaction. WGA inhibits various nucleocytoplasmic-transport pathways (Dargemont and Kuhn, 1992; Yoneda et al., 1987) by binding to O-glycosylated nucleoporins (Hanover et al., 1987). With limiting amounts of transportin or importin-9, no import of Rev was observed in the presence of WGA, demonstrating the specificity of the assay (Fig. 1B).

Importin-9 has not yet been described as an import receptor for HIV-1 Rev. As shown in Fig. 1C, importin-9, like transportin, bound to immobilised GST-Rev in the absence but not in the presence of RanQ69L. This Ran mutant abolishes the interaction of HIV-1 Rev with import receptors, and thereby demonstrates the specificity of the interaction. Note that a specific interaction of HIV-1 Rev with a recombinant import receptor does not necessarily qualify this receptor as a functional transport factor for the viral protein. In fact, all tested importins interacted to some extent with HIV-1 Rev (Arnold et al., 2006a), yet only two of them supported robust import in vitro. Together, our data suggest that, under conditions of limiting receptor concentrations, HIV-1 Rev is preferentially imported by transportin or importin-9 in vitro.

Because the HIV-1 Rev protein tends to stick to cytoplasmic structures, resulting in a variable cytoplasmic background, a reliable quantification of its nuclear-import efficiencies in vitro is hardly possible, in contrast to other substrates such as BSA-NLS. In light of the rather qualitative nature of the import assays for HIV-1 Rev in vitro, we focused our further analysis on intact cells.

Fig. 2. Transportin is the major import receptor for HIV-1 Rev in vivo. (A) HeLa cells were co-transfected with reporter constructs coding for GFP\(^{-}\)-M9core, GFP\(^{-}\)-cNLS or HA-Rev, as indicated, either with an empty vector (-M9M) or with a plasmid coding for Myc-MBP-M9M (+M9M) at a ratio of 1:7. Cells were stained for DNA and MBP-M9M (M9M), as indicated. The reporter proteins were either detected directly (GFP\(^{-}\)-M9 core, GFP\(^{-}\)-cNLS; top and middle panels) or by direct immunofluorescence (bottom panel). Cells were analysed by fluorescence microscopy. (B) HeLa cells were transfected with Rev-GFP either alone or in the presence of Myc-MBP-M9M and either empty vector, HA-transportin (HA-trn), HA-importin-9 (HA-imp 9) or HA-importin-β (HA-imp β), as indicated, at a ratio of 1:1:12. Cells were stained for MBP-M9M, import receptors and DNA, as indicated, and analysed by fluorescence microscopy. (C) GST-M9M was immobilised on beads and incubated with either transportin or importin-9 in the absence or presence of RanQ69L-GTP. Bound proteins were analysed by SDS-PAGE and immunoblotting using an anti-His antibody. The input (inp.) corresponds to 10% of the import receptors used in the binding reaction. (D) Mock-treated cells or those treated with an siRNA against transportin were transiently transfected with HA-Rev and NES (Rev aa 68-90)-GFP\(^{-}\)-M9core, stained for HIV-1 Rev and analysed by fluorescence microscopy. Scale bars: 10 μm.
Transportin is the main import receptor for HIV-1 Rev in HeLa cells

Transportin has been described as the specific import receptor for hnRNP A1 (Pollard et al., 1996), hnRNP M (Cansizoglu et al., 2007; Guttinger et al., 2004) and other proteins containing an M9 nuclear localisation signal (Imasaki et al., 2007; Lee et al., 2006). For importin-9, no specific import substrate is known, as its characterised binding partners can be imported by other transport receptors as well (Mühlhäusser et al., 2001). To investigate whether transportin and/or importin-9 could act as physiological import receptors of HIV-1 Rev in vivo, we made use of a recently described specific inhibitor of transportin-mediated import. The M9M peptide, a chimera of the M9 signals of hnRNP A1 and hnRNP M, has an increased binding affinity to transportin compared with the original nuclear localisation signals of the two hnRNP proteins, and can therefore act as a dominant-negative competitor of its natural NLS (Cansizoglu et al., 2007). The import of a fusion of green fluorescent protein (GFP) with the M9-core sequence [GFP–M9core (Iijima et al., 2006)] was significantly reduced in the presence of the M9M competitor (Fig. 2A, compare –M9M with +M9M). As a control, we used a reporter protein that contains a CNLS and is expected to be imported by the importin-α/β pathway. This GFP-CNLS fusion protein (GFP–CNLS) strongly accumulated in the nucleus, irrespective of the presence of the M9M competitor, reflecting the specificity of the M9M sequence for the transportin pathway. Upon transfection, the HIV-1 Rev protein mainly localised in the nucleus, with a strong enrichment in nucleoli, as shown before (Cochrane et al., 1990; Cullen et al., 1988). Strikingly, the coexpression of the M9M competitor efficiently reduced Rev nuclear localisation, resulting in a strong cytoplasmic accumulation of HIV-1 Rev. We next investigated whether overexpression of transportin or importin-9 could rescue the M9M-peptide-induced inhibition of the import of HIV-1 Rev. Nuclear import of HIV-1 Rev in the presence of M9M competitor was largely restored by overexpression of transportin or importin-9 (Fig. 2B), demonstrating that both transport receptors can mediate nuclear import of HIV-1 Rev in vivo. By contrast, the overexpression of importin-β, which had originally been described as the bona fide import receptor for HIV-1 Rev (Henderson and Percipalle, 1997; Truant and Cullen, 1999), could not stimulate nuclear import of the HIV-1 Rev protein in vivo. The functionality of HA–importin-β that was used for these experiments was confirmed using a CNLS-dependent reporter protein (data not shown) (Hutten et al., 2008). The M9M peptide was shown to bind specifically to transportin but not to importin-β (Cansizoglu et al., 2007). It is formally possible, however, that importin-9 also interacts with the M9M sequence. Given the strong inhibitory effect of the M9M peptide on the cellular localisation of HIV-1 Rev and the stimulation of Rev nuclear import by the coexpression of importin-9, we analysed whether the M9M sequence interacts with importin-9 as well (Fig. 2C). Transportin showed strong binding to immobilised GST-M9M and reduced sensitivity to RanQ69L, consistent with findings by Cansizoglu et al. (Cansizoglu et al., 2007). By contrast, no binding of importin-9 to GST-M9M could be detected, suggesting that the mislocalisation of HIV-1 Rev in the presence of M9M was indeed caused by inhibition of the transportin-dependent import pathway.

To demonstrate the dependence on transportin of the nuclear import of HIV-1 Rev by a second, independent approach, we analysed the localisation of HIV-1 Rev in cells that were depleted of transportin using an established small interfering RNA (siRNA) against transportin (Malnou et al., 2007). HIV-1 Rev was co-transfected with a transportin-dependent shuttling reporter (NES-GFP2-M9core) to identify cells that had reduced levels of transportin-dependent import. In control cells, the HIV-1 Rev protein as well as the shuttling reporter accumulated in the nucleus. In cells that had been treated with the siRNA against transportin, both the transportin-dependent shuttling reporter as well as the HIV-1 Rev protein showed a strong cytoplasmic accumulation (Fig. 2D). The localisation of an importin-α/β-dependent shuttling reporter (NES-GFP2-cNLS), by contrast, was not affected by the siRNA against transportin, demonstrating the specificity of the knock down (data not shown). Together, these data strongly suggest that transportin is the major import receptor for HIV Rev in vivo, at least in HeLa cells.

Nuclear import of HIV-1 Rev depends on Nup358

After identifying transportin as a major soluble factor in the nuclear import of HIV-1 Rev, we set out to analyse the components of the NPC that are required for the efficient import of this viral protein. Recently, we reported that Nup358, the main component of the cytoplasmic filaments of the NPC, is required for efficient importin-α/β-dependent transport (Hutten et al., 2008). To analyse whether Nup358 also plays a role in the nuclear import of HIV-1 Rev, we used our established RNA-interference protocol (Hutten et al., 2008), which leads to a specific and efficient reduction in Nup358 protein level. As shown in Fig. 3A,B, HIV-1 Rev is strongly enriched in the nucleus (N>C) in ~60% of control cells. This number decreased dramatically to ~10% in Nup358-depleted cells. Accordingly, the percentage of cells showing a cytoplasmic accumulation of HIV-1 Rev (N<C) increased from ~1% in control cells to ~20% in Nup358-depleted cells, and the number of cells with a more equal distribution between nucleus and cytoplasm (N=C) increased from 40% in control cells to ~60% in cells depleted of Nup358. Similar results were obtained using a different siRNA against Nup358 (supplementary material Fig. S1). For these experiments, HA-tagged HIV-1 Rev was used. Note that very similar results were obtained with untagged HIV-1 Rev (see below; also compare with Fig. 5). As Nup358 provides a binding site for sumoylated RanGAP at the NPC (Mahajan et al., 1997; Matunis et al., 1996), depletion of Nup358 leads to a concomitant loss of RanGAP from the nuclear pore, thereby increasing the pool of soluble, unsumoylated RanGAP (Hutten et al., 2008). Soluble RanGAP can efficiently promote importin-α/β-dependent nuclear import, but the role of nuclear-pore-associated RanGAP in transportin-mediated transport has not yet been investigated. Therefore, we tested whether the mislocalisation of HIV-1 Rev in the absence of Nup358 was caused by the depletion of RanGAP at the nuclear pore. Upon transfection of a Nup358 fragment that provides the RanGAP-binding site (HA–Nup358), RanGAP was efficiently removed from the NPC to a level comparable to that observed after Nup358 depletion (Hutten et al., 2008). The preferential nuclear localisation of the HIV-1 Rev protein, however, was not compromised in such cells (Fig. 3C), suggesting that pore-associated RanGAP is not required for nuclear import of HIV-1 Rev. Rather, the nucleoporin backbone of the Nup358-RanGAP complex appears to be responsible for the observed effects.

Theoretically, the increased cytoplasmic localisation of HIV-1 Rev in Nup358-depleted cells could result from either a decreased nuclear import or accelerated export. To distinguish between these two possibilities, we analysed the localisation of the HIV-1 Rev protein in the presence of leptomycin B (LMB), a very selective inhibitor for the CRM1-dependent export pathway (Wolff et al.,

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In the presence of LMB, HIV-1 Rev was found exclusively in the nucleus of mock-treated cells (Fig. 4A). In cells with reduced levels of Nup358, however, a significant fraction of the protein was retained in the cytoplasm when nuclear export was inhibited by LMB, indicating a reduced rate of nuclear import. To further investigate the effect of LMB on the subcellular localisation of HIV-1 Rev in the context of Nup358 depletion, we also used an established reporter construct consisting of HIV-1 Rev fused to the hormone-responsive domain of the glucocorticoid receptor (GR) and GFP, Rev-GR-GFP (RGG) (Love et al., 1998). At steady state, this reporter localised to the cytoplasm in both control and Nup358-depleted cells (Fig. 4B, –LMB). In control cells, its import was efficiently stimulated by the addition of the hormone analogue dexamethasone, as shown previously (Love et al., 1998). Import required an NLS as provided by the HIV-1 Rev part of the reporter, as a construct consisting of only the GR- and GFP-domain does not accumulate in the nucleus upon the addition of dexamethasone (Hutten et al., 2008). In Nup358-depleted cells, nuclear import of Rev-GR-GFP in the presence of dexamethasone was less efficient, compared with mock-treated cells (data not shown). In the absence of dexamethasone, the RGG-protein in B was detected directly. Scale bars: 10 μm.
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Recently, we showed that importin-β becomes rate-limiting for importin-α/β-dependent import in cells that had reduced levels of Nup358 (Hutten et al., 2008). Given our observation that HIV-1 Rev is mainly imported by transportin and that this is dependent upon Nup358, we next investigated whether transportin was rate-limiting for the nuclear import HIV-1 Rev in Nup358-depleted cells. As with importin-β (Hutten et al., 2008), the total-protein level for transportin remained unchanged after depletion of Nup358 (data not shown). We therefore analysed the localisation of HIV-1 Rev in control cells and in Nup358-depleted cells in the absence or presence of excess levels of transportin (Fig. 5A,B). As shown before, reduction of Nup358 strongly reduced the nuclear import of HIV-1 Rev. The nuclear localisation of Rev was restored to a level seen in mock-treated cells upon overexpression of transportin (Fig. 5A). Similarly, overexpression of importin-9 stimulated nuclear import of HIV-1 Rev in Nup358-depleted cells, confirming that importin-9 can be functional in mediating the nuclear import of HIV-1 Rev in vivo upon overexpression. Overexpression of importin-β, by contrast, did not rescue the import defect of HIV-1 Rev in Nup358-depleted cells (Fig. 5A, bottom panel), in agreement with our previous findings (Fig. 2B). Interestingly, we rather observed an inhibition of nuclear import in mock-treated cells that overexpressed HA–importin-β (Fig. 2B; compare with Fig. 5B). Import of a control substrate containing a classical NLS, by contrast, was promoted by overexpressed HA–importin-β, demonstrating its functionality (Hutten et al., 2008). The reason for import inhibition of HIV-1 Rev by overexpression of HA–importin-β remains to be investigated. Together, these results show that transport receptors become rate-limiting for the nuclear import of HIV-1 Rev in cells that have reduced levels of Nup358. Both transportin and importin-9, but not importin-β, function to restore the nuclear import of HIV-1 Rev in such cells. Our data also suggest that transportin and Nup358 are important cellular cofactors for a robust viral infection. Indeed, HIV-1 replication was reduced upon depletion of Nup358 (data not shown). This effect, however, clearly involved additional steps besides a function that is dependent on HIV-1 Rev (e.g. during the import of proviral DNA) and will therefore require further analysis. In agreement with our results, Nup358 was very recently identified in a genome-wide siRNA screen as a factor that is involved in nuclear import of the viral pre-integration complex (König et al., 2008).

Nup358 is a general factor in transportin-mediated nuclear import

Is the requirement for Nup358 in transportin-mediated import specific for HIV-1 Rev or does it apply to transportin-dependent nuclear import in general? To address this question, we made use of other dexamethasone-inducible reporter systems consisting of the hormone-responsive domain of GR and GFP fused to the M9 core domain (GR2–GFP2–M9core) or hnRNP M (GR2–GFP–hnRNP M). The import of both reporter proteins strictly depends on transportin, because the co-transfection of the dominant-negative inhibitor M9M strongly inhibited their nuclear import (supplementary material Fig. S2). Upon transient transfection, these reporter proteins localised to the cytoplasm in mock-treated as well as in Nup358-depleted cells. Upon addition of dexamethasone, they accumulated efficiently in the nucleus in control cells. By contrast, in cells with reduced levels of Nup358,
the number of cells showing efficient nuclear import was significantly reduced (Fig. 6). Again, similar results were obtained using a different siRNA for the depletion of Nup358 (supplementary material Fig. S3). To further analyse the function of Nup358, we compared the import rates of the GR2-GFP2-M9core reporter protein in mock-treated and Nup358-depleted cells. As shown in Fig. 7, cells depleted of Nup358 displayed a strongly reduced import kinetic after the addition of dexamethasone, compared with control cells. These data point towards a general function for Nup358 in the transportin-dependent import pathway.

Discussion
In this study, we identified transportin as the main receptor for the nuclear import of HIV-1 Rev in intact cells. Furthermore, we show that Nup358 promotes nuclear import of Rev and other transportin-dependent substrates.

Transportin is a major import receptor for HIV-1 Rev in HeLa cells
Several import receptors in addition to importin-β, namely transportin, importin-5 and importin-7, are known to mediate the nuclear import of HIV-1 Rev in vitro. The identity of the responsible import receptor in vivo, however, has not yet been determined. Here, we show for the first time that transportin is the main import receptor of HIV-1 Rev in intact HeLa cells. Additionally, we identified importin-9 as a functional import receptor for Rev in vitro, as well as in vivo, upon overexpression. The M9M sequence, which functions as a dominant-negative inhibitor of transportin-dependent import, did not bind to importin-9 in vitro but strongly reduced the nuclear localisation of HIV-1 Rev. In addition, specific depletion of transportin by RNA-interference led to strongly reduced nuclear accumulation of HIV-1 Rev. By immunoblot analysis, we found transportin and importin-9 to be expressed at similar levels in HeLa cells (5-10 μM; data not shown). As this concentration of importin-9 was not sufficient to compensate for the reduced levels of transportin, the cellular importin-9 pathway might have been saturated by dominant endogenous import cargos. Only upon overexpression was HA-tagged importin-9 able to promote nuclear import of HIV-Rev. In Jurkat T cells, a target cell line that more closely resembles the natural host of HIV-1, we detected much lower levels of importin-9 compared with transportin (data not shown).

Interestingly, overexpression of importin-β, the import receptor that was initially identified for HIV-1 Rev (Henderson and Percipalle, 1997; Truant and Cullen, 1999), did not promote import of HIV-1 Rev upon inhibition of transportin. This is in accordance with our recent data showing that an inhibitory antibody against importin-β did not inhibit the nuclear import of HIV-1 Rev in vitro (Arnold et al., 2006a). In the same study, we found that excess M9 substrate as a competitor for transportin-mediated import did not interfere with efficient nuclear import of HIV-1 Rev in vitro. At first glance, this seems to be at variance with our present data. However, under our in vitro conditions, other import receptors present at sufficient concentrations in the cytosolic extract (e.g. importin-5, importin-7 or importin-9) might substitute for transportin, despite not being capable of efficient transport in intact cells, in which many different nuclear transport events occur in parallel.

Together, these data indicate that transportin is the most likely import receptor for HIV-1 Rev in vivo. For a definite statement, however, it will be necessary to analyse nuclear import of this protein in the context of a viral infection of primary target cells.

Nup358 is required for the efficient nuclear import of HIV-1 Rev and of additional transportin-dependent substrates
The HIV-1 Rev protein is a shuttling protein that is required for the efficient export of partially spliced or unspliced viral RNA from the nucleus (Pollard and Malim, 1998). Under steady-state conditions, it is mainly localised in the nucleus with a strong enrichment in nucleoli (Cochrane et al., 1990; Cullen et al., 1988). Upon Nup358 depletion, however, we observed a dramatic relocalisation of HIV-1 Rev into the cytoplasm. Notably, the
mislocalisation of Rev appears to result from the Nup358 depletion and not from the accompanying relocalisation of RanGAP from the NPC into the cytoplasm. This shows that, as in importin-α/β-mediated import (Hutten et al., 2008), soluble RanGAP can fully substitute for pore-associated RanGAP in the transportin-dependent transport of Rev.

Formally, the enhanced cytoplasmic localisation could be due to enhanced export or reduced import of HIV-1 Rev upon Nup358 depletion. After inhibition of CRM1-mediated export by LMB, the HIV-1 Rev protein as well as the Rev-derivative RGG localised partially or completely in the cytoplasm of Nup358-depleted cells. In LMB-treated control cells, by contrast, these proteins strongly accumulated in the nucleus. This clearly shows that nuclear import is disturbed upon depletion of Nup358 and argues against accelerated CRM1-mediated export of the HIV-1 Rev protein. This is in accordance with previous results that show a slight inhibition rather than an acceleration of CRM1-dependent export upon Nup358 depletion (Bernad et al., 2006; Hutten and Kehlenbach, 2006).

Importantly, the observed dependence of transportin-mediated import on Nup358 is not an isolated phenomenon for HIV-1 Rev, but also applies to other well-characterised transportin-dependent substrates. Two different inducible reporter constructs, GR2-GFP2-M9 and GR2-GFP-hnRNP M, exhibited a significantly reduced nuclear import in cells depleted of Nup358. We have previously reported that M9-containing import substrates localise in the nucleus independently of Nup358 in vivo (Hutten and Kehlenbach, 2006). However, these observations were made with non-shuttling substrates. Such proteins will eventually accumulate in the nucleus after transfection, albeit with much slower kinetics, as seen for the GR2-GFP2-M9core reporter protein in Nup358-depleted cells (Fig. 7).

For cNLS-mediated nuclear import, we recently suggested a model in which the recycling of importin-β and formation of new import complexes is promoted by Nup358 (Hutten et al., 2008). We demonstrated that importin-β becomes rate-limiting in cells depleted of Nup358, leading to reduced import rates for cNLS-containing import substrates. Overexpression of the import receptor compensated for this effect. Does such a model also apply to transportin-dependent import? Indeed, overexpression of either transportin or importin-9 in Nup358-depleted cells restored the nuclear localisation of the HIV-1 Rev protein, showing that the transport receptors are rate-limiting for Rev import in Nup358-depleted cells. This suggests a function of Nup358 as an assembly and/or disassembly platform, not only for the recycling of importin-β but also for additional transport receptors such as transportin and possibly others. Nup358 is well known as a binding site for importin-β in complex with RanGTP (Delphien et al., 1997). To our knowledge, a similar mode of interaction for transportin with Nup358 has not yet been described, although a weak interaction of transportin with various nucleoporins has been observed using blot overlays (Bonifaci et al., 1997).

Shuttling of HIV-1 Rev is strictly required for the export of viral RNAs into the cytoplasm to allow the virus to proceed in its replication cycle (Felber et al., 2007; Pollard and Malim, 1998). Together, our data point to a dual role for Nup358 in the nucleocytoplasmic shuttling of HIV-1 Rev. In our model (Fig. 8), Nup358 supports Rev function by coordinating (1) CRM1-dependent export of HIV-1 Rev and (2) the formation of new Rev-transportin complexes for re-import into the nucleus. As a result, the individual players are prevented from diffusion into the cytoplasm by facilitating their recycling. The viral protein takes advantage of Nup358 as a docking station for cellular transportin-containing import complexes on their way into the nucleus. In light of our previous results concerning importin-β-dependent import, Nup358 appears to be a cytoplasmic nucleoporin that promotes multiple nuclear-import pathways.

Materials and Methods
Cell culture and transfections
HeLa-P4 cells (Charneau et al., 1994) were grown in DMEM (GIBCO) containing 4500 mg/l glucose, 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For expression of pcRev, F25-Rev-GFP, Rev-GR-GFP, HA-Rev, HA-transportin, HA-importin-9, HA-importin-β, HA-Nup358 (aa 2595-2881), Myc-MBP-M9M, GR-nes-GFP2-NLS and GR2-GFP-hnRNP M constructs, HeLa-P4 cells were transiently transfected using FuGENE (Roche) or Polyfect (Qiagen), according to the instructions of the manufacturer. To inhibit CRM1-mediated nuclear export, cells were incubated for 3 hours in the presence of 10 nM LMB [a kind gift from M. Yoshida (Kado et al., 1999)].

RNA interference
Cells were either mock-treated or transfected with siRNA against Nup358 (5'-CACAGAACAAGCCGGGUCAAA-3', corresponding to nucleotides 351-369; accession no. NM_006267) as described (Hutten and Kehlenbach, 2006). The siRNA against transportin was obtained from Santa Cruz (sc-514773) (Malnou et al., 2007). As control, an siRNA against firefly luciferase obtained from Dharmacon was used (siRNA GL2; 5'-CACGAACGCGGAAUCUCUGA-3', corresponding to nucleotides 153-173, accession no. AB261988.1).
Plasmids
The Myc-MBP-M9M (Cansizoglu et al., 2007), pRev (Malim et al., 1988), F25-Rev-GFP (Stauber et al., 1995), GST-Rev (Arnold et al., 2006a) and Rev-GFP (GGI) (Love et al., 1998) constructs have been described previously. The HA-Nup358 (2595-2981), HA-transportin and HA-importin-β and NES (Rev 68-90)/GR1-GFPp-eNLS constructs are described elsewhere (Hutter et al., 2008). The coding sequence of mouse importin-9 and of HIV-1 Rev were PCR-amplified and inserted into the Ncol-EcoRI and Clal-SpeI sites, respectively, of the EF-HA plink vector (Gasteiger et al., 2003).

For generation of the GR1/NES-GFP-M9core constructs, oligonucleotides coding for the hnRNP A1 M9 core (Iijima et al., 2006) (5'-AAATTTCCAAACCTTGGCGC-CATGAAAAAGAGAACATCTGAGACATCGACATGAATGGA-3' and 5'-GAATGTTTGAACACCGTGTTTCTTCTGTTGAAACCTCTCC-AGTGTCTCTGTAATAATCAGCT-3') were annealed and inserted into the EcoRI and SalI sites of the GR1/NESGFP-p-eNLS constructs (Hutter et al., 2008), thereby replacing the CNLS with the M9 core sequence. For the GR2/NES-GFP2-hnRNP M reporter, the coding sequence of hnRNP M was PCR amplified and inserted into the BglII and SalI sites of the GR1/NESGFP construct, replacing the GFP-NLS with hnRNP M.

His-YFP-Rev was obtained by PCR amplification of the coding sequence of HIV-1 Rev and ligation into the EcoRI and HindIII sites of the pT28-YFP vector (gift from F. Melchior, Department of Biochemistry I, University of Göttingen, Germany). For generation of GST-M9M, the M9M coding sequence of Myc-MBP-M9M was inserted into the BglII and NotI sites of the pGex-6P-1 vector. All constructs were verified by sequencing.

Protein purification
His-YFP-Rev was expressed in BL21-DE3-RIL cells upon induction with 0.25 mM IPTG at 18°C for 10 hours. Bacteria were lysed in Rev buffer (50 mM sodium phosphate, pH 6.5, 150 mM NaCl, 10 mM K2SO4, 4 mM Mg-mercaptoethanol and 1 μg/ml each of aprotinin, leupeptin and pepstatin). After centrifugation at 100,000 g for 40 minutes, the supernatant was incubated with Ni-NTA agarose (Qiagen) for 4 hours at 4°C. Beads were washed multiple times with Rev buffer and once with Rev buffer containing 10 mM imidazole and bound proteins were eluted with 300 mM imidazole in Rev buffer. Using PD-10 columns (Amersham Biosciences), the buffer was changed to 20 mM K2PO4, pH 7.4, 250 mM sucrose, 5 mM Mg(OAc)2, 200 mM KOAc, 1 mM EGTA and 2 mM DTT. GST-M9M was expressed in BL21(-DE3) cells and natively purified by single-step affinity chromatography using glutathione Sepharose beads (Amersham Biosciences), according to the instructions of the manufacturer. Importin-β (Chi and Adam, 1997), importin-5 (Jäkel and Görlich, 1998), importin-7 (Wohlwend et al., 2007), importin-9 (Mühlhäusser et al., 2001), importin-β (Mühlhäusser et al., 2001), and GST-Rev (Arnold et al., 2006a) were expressed as described previously. These proteins were dialysed against transport buffer (TPB; 20 mM HEPES-KOH, pH 7.3, 110 mM KOAc, 2 mM Mg(OAc)2, 1 mM EGTA, 2 mM DTT, and 1 μg/ml each of aprotinin, leupeptin and pepstatin), frozen in liquid nitrogen, and stored at -80°C.

Binding studies
GST-fusion proteins (5 μg) were immobilised on 20 μl glutathione Sepharose CL4B beads (Amersham Bioscience), which had been pre-incubated with 10 mg/ml BSA.

Antibodies
The mouse anti-HIV-1-Rev, goat anti-Nup358 and goat anti-RanGAP antibodies have been described previously (Hammerschmid et al., 1994; Hutter et al., 2008; Pichler et al., 2002). For the detection of HA-epitope-tagged proteins, either a monoclonal mouse anti-HA antibody (clone 12CA5) or a rabbit anti-HA antibody (Sigma) was used.

A total of 4 μg of recombinant transport receptors in 300 μl of binding buffer (50 mM Tris, pH 7.4, 200 mM NaCl, 1 mM MgCl2, 5% glycerol, 2 mg/ml BSA) were added. Where indicated, reactions included 1.3 μM RanQ69L-GFP. After 3-4 hours at 4°C, beads were washed four times with binding buffer. Bound proteins were eluted with SDS-sample buffer, subjected to SDS-PAGE and visualised by immunoblotting.

Nuclear-transport assay
To induce the import of the GR1/GFP-M9core and GR1/GFP-hnRNP M fusion protein, cells grown on poly-L-lysine-coated coverslips were treated with 5 μM dexamethasone (Sigma) for 10 minutes at 37°C, fixed and subjected to indirect immunofluorescence. For analysis of the kinetics of dexamethasone-induced import in vivo, mock-treated or siRNA-treated cells were plated on poly-L-lysine-coated LabTek-chambers (Nunc) and transfected with the construct GR1/GFP-M9core by the calcium-phosphate method (Ausubel et al., 1994). Cells were transfected to CO2-independent medium (Gibco) and nuclear import was induced with 5 μM dexamethasone. Images were taken every 10 seconds for 15 minutes on a Zeiss LSM 510-Meta confocal microscope (Carl Zeiss, Jena, Germany) equipped with an argon laser, a 63× Plan-Neofluar 1.3NA water-corrected objective and a temperature-controlled chamber, equilibrated to 37°C. The laser intensity was set to 2%. For every time point, nuclear and total cellular fluorescence were measured by the LSM image software. After background subtraction, the ratio of nuclear to total fluorescence was plotted against time.

For analysis of nuclear import in vitro, adherent cells were grown on coverslips, permeabilised with 0.01-0.015% digitonin in TPB and incubated for 30 minutes at 4°C or 23°C, with an ATP regenerating system (1 mM ATP, 2.8 mM creatine phosphate, 20 μM creatine phosphokinase; Sigma), 250 mM His-YFP-Rev, 2 mM Ran or RanQ69L, 2 mg/ml BSA, and 250 or 625 mM His-tagged import receptors, as indicated. As a specificity control, cells were preincubated with 200 μg/ml WGA (Sigma) for 10 minutes at 4°C.
Immunofluorescence

Immunofluorescence staining was essentially performed as described previously (Hutten and Kehlenbach, 2006). Cells were analysed by fluorescence microscopy using a Zeiss Axioskop2 microscope and AxioVision software. Images were processed using Adobe Photoshop. For the analysis of import efficiencies, cells were scored into the following categories: N=C (more protein in the nucleus than the cytoplasm), N>C (equal distribution of the reporter protein between nucleus and cytoplasm) and N<C (more reporter protein in the cytoplasm than the nucleus). P-values were obtained by performing a two-tailed, heteroscedastic t-test. P=0.05 was considered as biologically significant.

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