The nuclear import of H1 linker histones is mediated by a heterodimer of transport receptors, known as importinβ and importin7. Interestingly, both importins separately interact with H1, but only as a dimer they facilitate the translocation through the nuclear pore. We identified the H1 binding site of importin7, comprising two extended acidic loops near the C-terminus of importin7. The analysis of the H1 import complex assembly by means of isothermal titration calorimetry revealed that the formation of a receptor heterodimer in vitro is an enthalpy-driven process, whereas subsequent binding of H1 to the heterodimer is entropy-driven. Furthermore, we show that the importinβ binding domain of importin7 plays a key role in the activation of importin7 by importinβ. This process is allosterically regulated by importinβ and accounts for a specific tuning of the activity of the importinβ/importin7 heterodimer. The results presented here provide new insights into cellular strategies to even energy balances in nuclear import and point toward a general regulation of importinβ-related nuclear import processes.

The nuclear transport machinery represents one of the major transport systems in eukaryotic cells connecting the nucleus and the cytosol by bridging the nuclear envelope. Nuclear pore complexes (NPCs) are embedded in the nuclear envelope and provide the channels for nuclear transport. NPCs have a dual function; whereas solutes, ions and small macromolecules (20–40 kDa) are allowed to diffuse passively through the nuclear pore, larger macromolecules or such, whose transfer has to be tightly controlled, are transported by specific, soluble transport receptors in a signal-dependent manner (for review, see Refs. 1–4). The majority of known transport signals are specific recognition and translocation through the pore, the so-called co-import pathways. The nuclear import pathways known so far are subdivided in three classes. First, the receptor adaptor pathways with the well characterized example of the import of classical nuclear localization signal bearing substrates by importinβ (Impβ) and importinα (Impα). Second, the single receptor pathways, with a single importin directly binding its cargo as found in the import of small ribosomal proteins by the nuclear import receptors Impβ, transportin1 (Trn1), importin5 (Imp5), and importin7 (Imp7) (10) or the nuclear import of the parathyroid hormone-related protein PTHrP by Impβ (11). The third class applies a receptor pair, with each importin participating equally in substrate recognition and translocation through the pore, the so-called co-import pathways. A prominent example for such co-import pathways is the nuclear import of H1 linker histones. The functional import receptor for H1 is the Impβ-Imp7 heterodimer (12, 13). Only upon dimerization of Impβ and Imp7, a cooperative binding to H1 occurs (13). The ternary Impβ-Imp7-H1 complex then translocates through the nuclear pore and after translocation sticks to the nuclear basket. Upon binding of nuclear RanGTP to Impβ, the ternary complex is thought to dissociate into Impβ, which recycles into the cytosol in the RanGTP-bound state, and an Imp7-H1 dimer, which...
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travels on inside the nucleus to the DNA gyres. There, binding of RanGTP to Imp7 possibly represents the signal for transfer of H1 onto the chromatin. RanGTP-Imp7 is finally exported back to the cytosol completing the import cycle (10, 12).

Strikingly, substrates of co-import pathways tend to exhibit an extended, basic surface, that may be involved in unspecific interactions or aggregation in the cytosol as well as in the nucleus. As the need for coverage and protection of such basic regions during transport arises, only a pair of import receptors is thought to provide a joint substrate binding site large enough to also display a chaperoning activity (12). Interestingly, importins involved in co-import often are functional in other types of import, like Impβ and Imp7 as described above. Because the mechanisms of complex formation of Impβ and Imp7 and its specific role in H1 import remain elusive, the question arises of how such versatile molecules like Impβ and Imp7 accommodate to their different roles in the co-import pathway of H1 in comparison with common single receptor import. Hence, it is of high interest to thermodynamically characterize H1 import to see whether the import receptors Impβ and Imp7 modulate each other upon dimerization and how their cooperativity in binding H1 might be explained and, finally, to investigate if there are general thermodynamic indications on importin versatility.

EXPERIMENTAL PROCEDURES

Recombinant Protein Expression and Protein Purification—Human Impβ was expressed in *Escherichia coli* M15 (pREP4) with 300 μM isopropyl 1-thio-β-d-galactopyranoside at 18 °C overnight and purified as described previously (14). *Xenopus laevis* N-His10-Imp7 inserted into pQE-80 (a generous gift from D. Görlich, ZMBH Heidelberg, Germany) was expressed in *E. coli* BL21 (DE3) in 2YT medium with 0.4 mM isopropyl 1-thio-β-d-galactopyranoside, 30 mM K2HPO4, and 4% ethanol at 16 °C overnight. The purification was performed according to Baeuerle *et al*. (13). Impβ-N-396, Impβ-304-C were expressed and purified as described previously (15). Ran/TC4 was expressed in *E. coli* BL21 (DE3) RP in 2YT medium with 0.5 mM isopropyl 1-thio-β-d-galactopyranoside at 18 °C overnight and purified as described (16). The nucleotide exchange of GDP against GMPPNP, a nonhydrolyzable analog of GTP, was performed with the alkaline phosphatase method (17). Exchange efficiency was determined by HPLC.

The Impβ fragments Impβ-32-C, Impβ-127-C, Impβ-210-C, Impβ-N-641, Impβ-N-726, and Impβ-127–641 were subcloned from pQE-60-Impβ into the BamHI/SmaI sites of pGEX-6P-1 (GE Healthcare). Expression and purification were carried out in accordance with the protocol for glutathione S-transferase fusion proteins (GE Healthcare) with the addition of a final gel filtration with a Superdex 200 gel filtration column (XK 26/60, GE Healthcare) in analogy to full-length Impβ.

The *L. laevis* Imp7 fragments Imp7-N-916, Imp7-N-1001, Imp7-357-C, and Imp7-598-C were subcloned from pQE-9 into the MCS of pQE-80-Ndecahis (a generous gift from D. Görlich, ZMBH Heidelberg, Germany). These constructs were expressed and purified in analogy to the full-length protein.

The Imp7 fragments Imp7-598-C, Imp7-665-C, Imp7-824-C, Imp7-917-C, Imp7-1002-C, Imp7-876–1001, Imp7-917–1001, and Imp7-876–916 were subcloned from pQE-9-Imp7 into the BamHI/XhoI sites of pGEX-6P-1 (GE Healthcare). Expression and purification was analogous to that of the Impβ fragments. H10 was expressed and purified as described previously (13). Recombinant H1.2 from *B. taurus* was purchased from Axoxra, Germany.

H1.11L from *Gallus gallus* inserted into pET-13a (a generous gift from V. Ramakrishnan, Cambridge, UK) was expressed in *E. coli* BL21 (DE3) in 2YT medium with 0.4 mM isopropyl 1-thio-β-d-galactopyranoside. The cells were resuspended in a lysis buffer containing 50 mM Tris/Cl, pH 7.5, 100 mM NaCl, and protease inhibitors. After cell lysis in a microfluidizer 110S (Microfluidics, Newton, MA) and clarification by centrifugation at 30,000 × g, 4 °C for 45 min, the lysate was titrated to pH 8.8 and subsequently loaded onto a DEAE-Sepharose anion exchange column (GE Healthcare) in a buffer containing 50 mM Tris/HCl, pH 8.8, and 100 mM NaCl. The flow-through containing H1.11L was collected. After adjusting the NaCl concentration to 500 mM, the flow-through was loaded onto a SP-Sepharose cation exchange column (XK 26/20, GE Healthcare) in buffer A containing 50 mM Tris/HCl pH 8.8 and 500 mM NaCl. H1.11L was eluted from the column in a gradient from buffer A to buffer B (50 mM Tris/HCl pH 8.8, 1 mM NaCl) and finally purified with a Superdex S75 gel filtration column (XK 26/60, GE Healthcare) in a buffer containing 20 mM Tris/HCl, pH 7.3, and 100 mM NaCl.

Binding Assays—Pulldown assays on Ni-NTA-Sepharose. 2 nmol of His-tagged proteins were immobilized in binding buffer (20 mM Tris/HCl, pH 7.3, 300 mM NaCl, 2 mM β-mercaptoethanol) on 50 μl of HisTrap-Ni-NTA-Sepharose (GE Healthcare), and after copious washing in binding buffer with 40 mM imidazole, 4 nmol of the corresponding purified binding partners were applied to the Ni-NTA-Sepharose. Complexes were eluted with binding buffer containing 300 mM imidazole and analyzed via SDS-PAGE followed by Coomassie staining.

Pulldown assays on GSH-Sepharose (GE Healthcare) were performed equally with the following exceptions. Washing was done with binding buffer (20 mM Tris/HCl pH 7.3, 300 mM NaCl), and elution was performed in binding buffer containing 20 mM reduced glutathione.

The binding assays for gel filtration analysis of Impβ/Imp7 interactions were prepared in gel filtration buffer containing 20 mM Tris/HCl pH 7.6 and 100 mM NaCl. For interaction analysis of import receptors with H1 the salt concentration in the gel filtration buffer was increased to 300 mM NaCl. To achieve a good separation of formed complexes from the monomers, the protein quantities were 2–4 nmol of the larger binding partners; the smaller ones were each added in a 1.5-fold molar excess. Complex formation was tested on an analytical Superdex 200 column (S200 10/300 GL, GE Healthcare) and analyzed by SDS-PAGE with subsequent Coomassie staining.

In Vitro Nuclear Import Assays and Immunofluorescence Detection—Preparation of the fluorescent import substrates 4z-rpl23a and 6z-H10 was done as described (10, 13). The permeabilization of HeLa cells, the preparation of the energy regenerating system, and the Ran mix were done
according to the protocol of Adam et al. (18) and modifications described by Jäkel and Görlich (10). Recombinant nuclear import receptors were applied in concentrations as indicated in the figures. As a positive control, 10 μl of reticulocyte lysate were added to the import assay; the negative control lacked any of the recombinant import factors and the reticulocyte lysate. Import was allowed to progress for 15 min at 37 °C (import of rPL23a) and 30 min at 30 °C (import of H1) before the cells were washed and fixed in 3.7% formaldehyde. For immunofluorescence detection of His-tagged proteins the nuclei were subsequently permeabilized with 0.5% Triton X-100 and washed. Upon blocking with 0.2% fish gelatin the cells were incubated with a α-His antibody from Mus musculus and after copious washing incubated with the secondary antibody, a α-mouse antibody (Capra hircus) labeled with Alexa 594. Finally nuclear import was monitored by fluorescence microscopy with a Zeiss Axioskop20 microscope. Confocal laser scanning microscopy was done with a Leica DM IRE2 microscope, a Leica TCS SP2 spectral detector, and Leica confocal software. The immunofluorescence signals of 40 randomly chosen nuclei per experiment were quantified with ImageJ. For the comparison of different import assays, the immunofluorescence signal of the negative control was subtracted from all other values before the final evaluation.

**Isothermal Titration Calorimetry**—For determination of binding isotherms of Impβ, Imp7, and RanGDPNP, all proteins were in a buffer containing 20 mM Tris/HCl, pH 7.3, 100 mM NaCl, and 1 mM MgCl₂. The isotherms were recorded at 25 °C. For isothermal titration calorimetry (ITC) experiments with H1.11L and for determination of the specific heat capacity of the Impβ-Imp7-interaction (additional binding isotherms were recorded at 15 °C and 10 °C) all proteins were in a buffer containing 20 mM Tris/HCl, pH 7.3, 200 mM potassium acetate, 20 mM potassium phosphate, and 1 mM MgCl₂. For H1.11L interactions all isotherms were recorded at 10 °C. ITC experiments were carried out with a VP-ITC microcalorimeter (Microcal). The protein concentrations were 5 μM in the sample cell and 50–100 μM in the injection syringe. Data were analyzed with Origin 7.0, and the fitting curves were calculated according to one set of binding site models.

**RESULTS**

Previous studies revealed the Impβ-Imp7 heterodimer to be the only functional import receptor for H1 linker histones (12, 13). The binding sites on Impβ for Imp7 and H1₀ have already been identified (12) and include HEAT repeats 4–9 (Imp7) and HEAT repeats 6–19 (H1₀). They overlap and comprise the central part of Impβ. The interaction site of Imp7 for Impβ has also been identified previously (13). The H1 binding site of Imp7 was unknown as was the binding site of Impβ for H1 subtypes other than H1₀. Because evidence was given that during H1 import Impβ/Imp7 might not only act as import receptors but as chaperones as well (13, 19), the question arose as to whether the properties of the binding sites of Impβ-Imp7 for the linker histone H1 support the hypothesized chaperoning activity of the Impβ-Imp7 heterodimer during nuclear import. An energy characterization of the formation of a trimeric Impβ-Imp7-H1 complex was of high interest as well to investigate if there are energy requirements for a receptor dimer in H1 nuclear import.

In this study numerous deletion mutants of both Impβ and Imp7 were constructed to locate their binding sites for H1.2 and H1.11L, respectively, and to investigate the effects of dimerization of Impβ and Imp7 on subsequent H1 binding. Additionally, the energy requirements of trimeric Impβ-Imp7-H1 complex formation were characterized by ITC.

The newly designed Impβ deletion constructs used in this study comprise fragments with incomplete binding sites either for Ran or Imp7 and H1₀ (see Fig. 1A). In contrast to most previous studies, they were designed in accordance with a crystal structure of Impβ (Ref. 8 and supplemental Fig. 1), with special attention to the HEAT repeat organization of the molecule. Only complete HEAT repeats were deleted, to maintain correct folding of the resulting fragments. The Impβ deletions should allow for the identification of the H1.2 and H1.11L binding site of Impβ. The construction of fragments of Imp7 focused on N-terminal deletions (see Fig. 3A), as sequence analysis revealed two extended and rather disordered acidic regions (aa 882–912 and 927–957) at the C terminus of Imp7, which may be involved in substrate recognition. For a complete list of all tested constructs, see supplemental Fig. 2.

**Binding Sites of Impβ for RanGTP and Imp7 Overlap but Allow for Simultaneous Binding**—Although the binding sites of Impβ for RanGTP and Imp7 have been elucidated previously, showing an overlap of both sites within Impβ (5, 7, 12), the question of whether binding of Impβ to Imp7 and RanGTP are mutually exclusive in vitro has not been addressed so far.

To elucidate the organization of binding sites on Impβ, the binding capabilities of Impβ fragments for RanGTP and Imp7 or Imp7_1002-C, containing solely the importinβ binding domain of Imp7 (IBB₇, aa 1008–1038), were tested in pulldown assays via the recovery of Imp7 and Ran by the immobilized Impβ constructs. Gel filtration chromatography was applied to confirm the stability of complexes observed in pulldown experiments (see Figs. 1 and 3). The analysis of truncated Impβ fragments confirms the previously published minimal binding site for Imp7, represented by an N-terminal-located part of Impβ comprising HEAT repeats 6–9 (aa 210–396, Fig. 1). Further N-terminal deletions abolish binding to Imp7_1002-C completely, as is demonstrated for Impβ_304-C (Fig. 1, A and B).

Therefore, a prominent overlap of the binding sites of Impβ for Imp7 and RanGTP, comprising aa 210–396, could be confirmed. Because RanGTP was previously shown to dissociate the Impβ-Imp7 heterodimer, the question was whether RanGTP achieves this by a competitive displacement mechanism, pointing to interactions of Imp7 and RanGTP with identical amino acid side chains of Impβ. Surprisingly, in our hands RanGDPNP did not necessarily dissociate the Impβ-Imp7 heterodimer (Fig. 2). In fact, simultaneous binding of Imp7 and RanGTP to Impβ was observed in gel filtration experiments, where a significant binding of a purified Impβ-RanGDPNP heterodimer to Imp7 could be verified. In this experiment an Imp7 construct with N-terminal His tag was used which abolishes binding of Imp7 to RanGDPNP (not shown). This suggests that simultaneous binding of RanGTP and Imp7 to Impβ is possible in vitro, yet probably with reduced affinity.
Any H1 Subtype Interacts with the Same Binding Site on Impβ—With respect to the three-dimensional structures of Impβ (5, 8), several Impβ fragments with intact HEAT repeats were tested in binding to H1. Because the question remained unanswered of whether the contribution of RanGTP to any H1 import complex disassembly is identical, various H1 subtypes were applied in the binding experiments. Despite previous findings, where a stable complex formation with H1 was only demonstrated for an Impβ/Imp7 heterodimer and the single import receptors were shown to bind H1 with strongly reduced affinity (12), a significant and stable complex formation of full-length Impβ with H1.2 could be observed in the absence of Imp7 (Fig. 1C). Binding of Impβ to H1.2 is mediated by a large portion of Impβ involving HEAT repeats 4–14 (aa 127–641, Fig. 1, A and C), as is shown for Impβ_127–641. Further deletions of either the N terminus or the C terminus, represented by Impβ_N-396 and Impβ_210-C, respectively, abolish H1.2 binding completely. Thus, the binding sites for both Imp7 and H1.2 on Impβ do overlap (see Fig. 4, upper), implying a cooperative binding mode of the Impβ/Imp7 heterodimer and H1.2. In addition, the H1.2 binding site is very similar to the one for H1 (12), pointing to a common mechanism of binding of Impβ to several H1 subtypes. Supporting this hypothesis, a difference in binding of several subtypes of H1 to Impβ could not be observed when repeating the experiments with H1 and H1.1L from G. gallus (data not shown). All tested histones display significant similarities to each other with sequence identities between 43% (H1.2) and 41% (H1.1L) in comparison to H1 (see supplemental Fig. 3). Consecutively, a similarity in the primary structure corresponds to similar if not the same requirements for binding sites on Impβ/Imp7.

Moreover, the RanGTP binding site of Impβ (HEAT repeats 1–8 and 14–15, aa 20–378 and 639–682) (5, 7) overlaps with both Imp7 and H1 binding sites (see Fig. 4), as published previously (12). In contrast to the interaction between Impβ and RanGTP, which allowed for simultaneous binding of Imp7, a simultaneous binding of H1.2 and RanGTP to Impβ could not be observed (data not shown), implying a mutual exclusivity of binding to Impβ, and therefore, at least in part identical requirements for interaction sites on Impβ. This probably includes the acidic loop within HEAT 8 of Impβ (aa 333–343), for this loop has been reported to be involved in a competitive displacement of substrates bound to Impβ by RanGTP.
Because Imp7 lacks an acidic loop in a comparable position but instead displays two large, acidic loops near its C terminus, we next determined their role in H1 nuclear import.

**H1 Binding by Imp7 Is Conferred by Its Acidic C Terminus**—Because the H1 interaction site of Imp7 has not been identified previously, deletion mutants of Imp7 were designed, allowing for the identification of the binding site on Imp7 for the linker histone H1.2. Their ability of binding to H1.2 was analyzed via pulldown assays and gel filtration experiments. As a control for correct folding of the C-terminal fragments, their binding capability to Imp\(^{-}\)H9252 was also tested (data not shown), as IBB\(_7\) is located at the very C terminus (Fig. 3\(^A\) and Ref. 13).

The C terminus of Imp7 including two prominent acidic loops (aa 882–912 and 927–957, respectively) could be identified to be crucial in mediating H1.2 binding (Fig. 3). The minimal H1.2 binding site of Imp7 in pulldown assays comprises aa 824–1001. Both Imp7\(_{824-C}\), lacking the Ran interaction site, and Imp7\(_{N-1001}\), lacking IBB\(_7\), are able to bind H1.2 (Fig. 2B). However, stable complex formation of Imp7\(_{824-C}\) and H1.2 could not be observed in gel filtration experiments (data not shown). A level of H1.2 binding comparable with full-length Imp7 requires aa 665–1001 (Fig. 4). Again, as already reported above in the analysis of Imp\(\beta\), a difference in binding several subtypes of H1 by Imp7 could not be observed (data not shown). Interestingly, the IBB\(_7\) domain (1008–1038) is located in close vicinity to the H1 binding site, yet its presence is dispensable for H1 binding to Imp\(\beta\). This implies a linear organization of binding sites on Imp7, in contrast to Imp\(\beta\), indicating cooperative effects of IBB\(_7\) on H1 binding by Imp7 upon heterodimerization with Imp\(\beta\). To further characterize the Imp\(\beta\) and Imp7 fragments, not only binding to H1 was investigated but nuclear import of H1 as well.

**The IBB Domain of Imp7 Induces Cooperativity of Imp\(\beta\) and Imp7 in Binding H1**—The import capability of truncated fragments of both Imp\(\beta\) and Imp7 was tested in *in vitro* nuclear import assays to elucidate the specific role of each receptor during H1 import. Concerning nuclear import of substrates of single receptor pathways, the activity of an IBB\(_7\)-deficient fragment of Imp7, Imp7\(_{N-1001}\), remains unaffected, as this fragment is fully operational in nuclear import of the small ribosomal protein rpL23a (Fig. 5\(A\), panel 5), which is known to be a common substrate for such nuclear import pathways (10). When focusing on H1 import, previous findings that only a dimer of Imp\(\beta\) and Imp7 is able to function as import receptor for H1 (13) could be confirmed, as none of the import receptors is capable of mediating nuclear import alone (Fig. 5\(B\), panels 3 and 4). Both importins need to be present to regain H1 import capability (Fig. 5\(B\), panel 5). In addition, the heterodimerization before H1 binding is apparently necessary to gain full import competence; adding Imp7 to a preincubated Imp\(\beta\)-H1 complex
Imp7 constructs | scheme | Impβ binding | H1 binding
---|---|---|---
Imp7 fl | RanGTP | +++ | +++
Imp7_N-916 | | - | -
Imp7_N-1001 | | - | +++
Imp7_598-C | | +++ | ++
Imp7_665-C | | ++ | +
Imp7_824-C | | +++ | -
Imp7_1002-C | | +++ | -
Imp7_876-1001 | | - | -
Imp7_917-1001 | | - | -
Imp7_876-916 | | - | -

**FIGURE 3.** The H1 binding site of Imp7 is located between aa 665 and 1001 including the C-terminal acidic loops of Imp7 and does not overlap with IBB. A, schematic representation of the soluble Imp7 fragments and their qualitative binding capacities to Impβ and H1. Four constructs, namely Imp7_598-C (aa 598–1038), Imp7_665-C (aa 665–1038), Imp7_824-C (aa 824–1038), and Imp7_1002-C (aa 1002–1038) contain the Impβ binding site (aa 1008–1038, in the following referred to as IBB), thus, putatively allowing dimerization with Impβ, whereas five other deletion constructs do not: Imp7_N-1001 (aa 1–1001), Imp7_876–1001 (aa 876–1001), Imp7_N-916 (aa 1–916), Imp7_917–1001 (aa 917–1001), and Imp7_876–916 (aa 876–916). The latter three additionally lack one or both of the acidic loops of full-length Imp7. The ability of the fragments to interact with Impβ and H1 is indicated by + and – in comparison with wild-type level of binding of full-length Imp7 (+ ++). Wild-type levels of H1 binding require amino acids 665–1001. The acidic loops of Imp7 are both indispensable for binding H1 and are solely involved in the interaction with H1 but not in binding either RanGTP or Impβ. B, SDS-PAGE of binding assays of Imp7 fragments and H1.2. The interaction of glutathione S-transferase-fused fragments of Imp7 with H1.2 was tested in pulldown experiments as described under “Experimental Procedures.” The lanes show the elution fractions. The interaction of the other Imp7 fragments with H1.2 was analyzed by gel filtration. Here the peak fractions of the Imp7 fragments are shown. Where co-eluted, H1.2 is marked by an asterisk; the masses of the molecular weight standard (MW) are as indicated.

(Fig. 5B, panel 6) does not rescue H1 import capability of Impβ-Imp7. Conversely, the addition of Impβ to a preformed Imp7-H1 complex allows the reconstitution of H1 import (Fig. 5B, panel 7), yet with a slightly diminished import capability in comparison to the full-length proteins as the receptor concentrations had to be increased in that experiment (0.5 μM, full-length proteins 0.2 μM). Notably, a complete loss of H1 import capability is lost as well. However, an increase of the receptor concentration allows a reconstitution of H1 import. An immunofluorescence detection with an anti-His antibody (Fig. 6B) shows that His-Imp7C598 accumulates in the nucleus, demonstrating that this deletion is trapped in the nucleus. A quantification of the immunofluorescent signals (Fig. 6C) backs this observation. When subtracting the mean background signal of activity can be observed when applying Impβ and Imp7_N-1001 (Fig. 5B, panel 8) despite the findings that heterodimerization of Impβ and Imp7 is dispensable in in vitro reconstitution assays. Thus, the formation of an Impβ-Imp7 heterodimer is a prerequisite for H1 import in vivo, supporting previous data (13). For further investigation of the specific role of each importin in H1 nuclear import, the reconstitution of H1 import was tested with Impβ_127–641 and Imp7_598-C. Impβ_127–641 has an incomplete RanGTP interaction site (20) but comprises any other domain needed for H1 import, namely the interaction sites for Imp7, H1, and the nucleoporins of the NPC. Imp7_598-C comprises the binding sites for H1 and Impβ but lacks the Ran binding site completely. The results show that even at high concentrations of the import receptors a co-import of H1 by Imp7 and Impβ_127–641 cannot be reconstituted (Fig. 5B, panels 9 and 10). Because Nup153, a nucleoporin at the nuclear basket of the NPC, is known to be the final binding partner of Impβ before its release into the nucleus upon RanGTP binding to Impβ, this might be due to an initial blocking of the NPC at the nuclear basket in the very first round of import. Confocal laser scanning microscopy (Fig. 6A) reveals a prominent staining of the nuclear envelope. Although a precise localization of the fluorescence signal is not possible, the rim staining points to a clogging of the NPC. Thus, Ran binding to Impβ and dissociating the Nup153:Impβ:Imp7-H1 complex is indispensable for termination of H1 import. When performing the identical experiment with Impβ and Imp7_598-C (Fig. 5B, panels 11 and 12) applying low receptor concentrations, the import
the negative control from the mean values of the signals of the reconstitution experiments (see supplemental Table 1), the resulting noise-reduced values show that only Imp\(_7\)\(_{598}\)-C is subject to nuclear trapping. This is demonstrated by the extraordinarily high nuclear immunofluorescence signal when applying 2 \(\mu\)M Imp\(_{1-14}\)/HiHis-Imp\(_7\)\(_{598}\)-C (1.71-fold in comparison to 2 \(\mu\)M Imp\(_{1-14}\)/HiHis-Imp\(_7\), Fig. 6C). In contrast to that, all other experiments including the control experiment with Imp\(_{1-14}\)/HiHis-Imp\(_7\)\(_{598}\)-C show no increase of nuclear immunofluorescence. Consequently, Imp\(_7\)\(_{598}\)-C is retained efficiently inside the nucleus. Thus, raising the concentration of Imp\(_7\)\(_{598}\)-C causes an increase of the immunofluorescence signal but in terms of import compensates for the nucleoplasmic trapping. Therefore, the trimeric import complex of Imp\(_{1-14}\)/HiHis-Imp\(_7\)-H1 is dissociated into RanGTP-Imp\(_{1-14}\) and Imp\(_7\)-H1. In summary, the interaction between Imp\(_{1-14}\) and RanGTP terminates the H1 nuclear import process, whereas the RanGTP binding site of Imp\(_7\) is dispensable. As in the case of IBB\(_7\), the H1 nuclear import capability is lost; the heterodimerization of Imp\(_{1-14}\) and Imp\(_7\) before H1 binding is a prerequisite for H1 import.

**Thermodynamic Analysis of the Interactions of Imp\(_{1-14}\), Imp\(_7\), H1, and RanGTP**—To answer the questions concerning possible thermodynamic reasons for an Imp\(_{1-14}\)/Imp\(_7\) heterodimer in H1 nuclear import, ITC was used to characterize the binding properties of the different complexes by determining their binding constants.

At first, the heterodimerization of Imp\(_{1-14}\) and Imp\(_7\) was investigated by characterizing the interaction of Imp\(_{1-14}\) and Imp\(_7\)\(_{1002}\)-C, for reasons of convenience referred to as IBB\(_7\) (Figs. 7A and 8A). This fragment was chosen for the interaction studies because the thermodynamic characteristics of full-length Imp\(_7\) and IBB\(_7\), in binding to Imp\(_{1-14}\) did not significantly change besides a slightly reduced affinity of full-length Imp\(_7\) to Imp\(_{1-14}\) (data not shown). The heterodimerization of Imp\(_{1-14}\) and IBB\(_7\) is an exothermic process with a binding enthalpy of about \(-67\) kJ/mol at 25 °C, a rather high affinity (\(K_D = 71\) nM), and an entropy change \(\Delta S\) of \(-89.6\) J/(K·mol). The relation between Gibbs free energy and the changes in enthalpy and entropy reveals that dimerization of Imp\(_{1-14}\) and Imp\(_7\) is enthalpy-driven despite a significant entropy-enthalpy compensation, which is typical for protein-protein interactions: \(\Delta G = \Delta H - T\Delta S = -66.9\) kJ/mol + 298 K × 89.6 J/(K·mol) = -40.2 kJ/mol.

The entropy decrease is completely compensated by the enthalpy change. Because the binding isotherm of Imp\(_{1-14}\) and IBB\(_7\) at 15 °C displays a binding enthalpy of \(-54\) kJ/mol (not shown), the interaction has a specific heat capacity \(\Delta C_p\) of \(-1.3\) kJ/(mol·K). The decrease of \(\Delta C_p\) with decreasing temperature points to the involvement of hydrophobic interactions between Imp\(_{1-14}\) and Imp\(_7\). When comparing the binding isotherms of Imp\(_{1-14}\) fragments with IBB\(_7\), (Figs. 7A and 8A), a decrease of affinity of Imp\(_{1-14}\) deletions to IBB\(_7\), is coherent with extending N- and C-terminal deletions of Imp\(_{1-14}\) (Fig. 7A), surprisingly including Imp\(_{127}\)-C (\(K_D = 258\) nM) and Imp\(_{210}\)-C (\(K_D = 216\) nM), which apparently bound to Imp\(_7\) as efficiently as full-length Imp\(_{1-14}\) in pulldown and gel filtration experiments (Fig. 1B). In the case of the interaction between Imp\(_{210}\)-C and IBB\(_7\), the affinity dramatically decreases from 71 nM (full-length Imp\(_{1-14}\)) to 1.86 \(\mu\)M (Imp\(_{210}\)-C, Fig. 7A). Thus, for dimerization of Imp\(_{1-14}\) and Imp\(_7\) at the level of the full-length proteins, the complete N terminus of Imp\(_7\) needs to be present. When deleting the five C-terminal HEAT-repeats 15–19 (aa 642–876), the affinity of Imp\(_7\) to IBB\(_7\)-C remains unaffected compared with the binding of the full-length protein, as is displayed by Imp\(_7\)\(_N\)-641. In fact, the affinity even increases (Fig. 7A, Fig. 8A).

Within Imp\(_{1-14}\) the interaction sites for Imp\(_7\) (HEATs 1–14 for wild-type binding) and RanGTP (5, HEATs 1–15) show a prominent overlap. Because the gel filtration analysis of the interaction between RanGTP-Imp\(_{1-14}\) and Imp\(_7\) revealed simultaneous binding of both Imp\(_7\) and RanGTP to Imp\(_{1-14}\), ITC was used to check for differences of the binding constants of Imp\(_{1-14}\) and IBB\(_7\), and a preincubated RanGDPNP-Imp\(_{1-14}\) complex and IBB\(_7\), respectively, to elucidate the influence of RanGTP on the Imp\(_{1-14}\)/Imp\(_7\) heterodimer (Fig. 7B). The binding isotherms demonstrate a 7-fold reduced affinity of IBB\(_7\), to Imp\(_{1-14}\)/RanGDPNP with regard to Imp\(_{1-14}\) in the absence of Ran. Although monomeric Imp\(_{1-14}\) binds IBB\(_7\), with a \(K_D\) value of 71 nM, the affinity of the Imp\(_{1-14}\)/RanGDPNP complex to IBB\(_7\), is significantly lowered, with a \(K_D\) value of only about 500 nM (Fig. 7A). The corresponding binding enthalpies do not differ, and although a competition of IBB\(_7\) and RanGTP for parts of the binding sites on Imp\(_{1-14}\) cannot be excluded, the similar \(\Delta H\) values might indicate that RanGTP binding to Imp\(_{1-14}\) does not reduce the interaction sites on Imp\(_{1-14}\) for IBB\(_7\). Remarkably, the entropy significantly changes from \(-89.6\) J/(K·mol) (Imp\(_{1-14}\)/RanGDPNP + IBB\(_7\)) to \(-177.1\) J/(K·mol) (Imp\(_{1-14}\)/RanGTP + IBB\(_7\)).

In conclusion, the results suggest that the binding sites of Imp\(_{1-14}\) for Imp\(_7\) and RanGTP are parallel-organized, meaning that they do not only overlap but allow for simultaneous binding of Imp\(_7\) and RanGTP to Imp\(_{1-14}\) as well. Yet due to a significant reduction of the affinity of Imp\(_{1-14}\) to Imp\(_7\) upon binding RanGTP, a dissociation of the heterodimer at the nuclear basket caused by RanGTP binding appears likely. An allosteric effect of RanGTP on the dissociation of Imp\(_{1-14}\) and Imp\(_7\) is plau-

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**FIGURE 4. Spatial organization of the binding sites of Imp\(_{1-14}\) and Imp\(_7\).** The binding sites of Imp\(_{1-14}\) for Imp\(_7\) and H1 are parallel-organized, meaning that they overlap and allow for simultaneous binding. In contrast, the binding sites of Imp\(_7\) for RanGTP (RanBS\(_7\)), Imp\(_{1-14}\) (IBB\(_7\)), and H1 are sequentially organized and non-overlapping.

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**TABLE 1.** Binding sites of Imp\(_{1-14}\) and Imp\(_7\) to RanGTP and H1. The binding sites of Imp\(_{1-14}\) for Imp\(_7\) and H1 are parallel-organized, meaning that they overlap and allow for simultaneous binding. In contrast, the binding sites of Imp\(_7\) for RanGTP (RanBS\(_7\)), Imp\(_{1-14}\) (IBB\(_7\)), and H1 are sequentially organized and non-overlapping.

| Binding | Imp\(_{1-14}\) Binding Sites | Imp\(_7\) Binding Sites |
|---------|----------------------------|-------------------------|
| RanGTP  | aa 20-582 (HEATs 1-15)      | aa 210-396 (HEATs 6-9)  |
| H1      | aa 127-641 (HEATs 4-14)     | aa 665-1001             |
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A  

**FIGURE 5. The IBB₂ domain of Imp7 is only required for receptor-heterodimer import.** *A*, in vitro nuclear import assays with rpl23a show that the purified importins Impβ and Imp7 are functional (panels 3 and 4). Imp7_N-1001 (panel 5) displays a nearly wild-type level of import capability when compared with full-length Imp7. DAPI, 4',6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate. B, H1 import can only be reconstituted with Impβ and Imp7 when both receptors interact with different, non-overlapping regions of the receptor-heterodimer complex (panels 6 and 7). Whereas import of H1 by Impβ and Imp7 can only be reconstituted with Impβ and Imp7, H1 import cannot be reconstituted with Imp7 and Impβ_127–641 (panels 8 and 9). The import of H1 by Impβ and Imp7 can be achieved with increasing receptor concentrations (retic, reticulocyte lysate). Receptor concentrations are as indicated.

sible. The binding sites for Imp7 and H1 on Impβ overlap but allow simultaneous binding as well. Therefore, we next addressed the question of if the specific organization of binding sites on Impβ for Imp7 and H1 accounts for cooperativity of Impβ·Imp7 in H1 binding, or due to the linear organization of Imp7, if cooperativity of the heterodimer is induced by a conformational switch in Imp7.

**Heterodimerization of Impβ and Imp7 Partly Balances the Energy Requirements for H1 Binding in Vitro**—To understand the interactions within the trimeric Impβ·Imp7·H1 complex, the binding constants of each of the participating subcomplexes were determined by isothermal titration calorimetry in a buffer containing relatively high amounts of salt ions (see “Experimental Procedures”). This buffer was required to circumvent precipitation of the ternary Impβ·Imp7·H1 complex and truncated versions thereof at the moment of complex formation. The experiments reveal that H1 binding is an endothermic process (Fig. 7C), in contrast to heterodimerization of Impβ and Imp7. An increase of the energy of about 309 J/K·mol (Imp7 + H1) and 360 J/K·mol (Impβ + H1) points toward a compensation of the binding enthalpy by an entropy-increasing displacement of salt counterions, which are bound on the surface of H1 by the importins. When titrating each of the import receptors with H1, it becomes evident that the affinities of Impβ and Imp7 to H1 are very similar to each other, with \( k_d \) values of 336 nM (Impβ) and 273 nM (Imp7), respectively (Fig. 7B). The affinity of a pre-incubated Impβ·Imp7 heterodimer to H1 is about 2–3 times higher in comparison to the single receptors (\( k_d \) value of 126 nM, Fig. 7B).

Notably, the enthalpy of the interaction between Impβ·Imp7 and H1 (\( \Delta H = 58.7 \text{ kJ/mol} \)) equals the arithmetic mean of the enthalpies of Impβ + H1 and Imp7 + H1: 

\[
\Delta H = \frac{\Delta H_{\text{Impβ+H1}} + \Delta H_{\text{Imp7+H1}}}{2} = (66.7 + 50.0) \text{ kJ/mol} \times 2 \approx 58.4 \text{ kJ/mol} = \Delta H_{\text{Impβ·Imp7+H1}}.
\]

The same is also true for the entropy changes:

\[
\Delta S_{\text{Impβ·Imp7+H1}} = 339.9 \text{ J/(K·mol)} / (\Delta H_{\text{Impβ·Imp7+H1}} - \Delta H_{\text{Impβ+H1}}) = \frac{339.9}{126} = 2.7 \text{ J/(K·mol)}.
\]

Considering the stoichiometry of binding of the single receptors to H1, which is 2:1 (\( n = 0.5 \)) in both cases, the binding enthalpies and entropies have to be halved to determine the enthalpies of one importin binding to H1. In that case the sums of the single enthalpies and entropies, respectively, equal the enthalpy and entropy of the heterodimer binding to H1. This implies that both receptors interact with different, non-overlapping regions of H1.

Notably, the process of H1 binding by Impβ·Imp7 *in vitro* is endothermic but exergonic: 

\[
\Delta G = \Delta H - T\Delta S = 58.7 \text{ kJ/mol} - 283 \times 339.9 \text{ J/(K·mol)} = -37.5 \text{ kJ/mol}. 
\]

Thus, the formation of the trimeric Impβ·Imp7·H1 complex in vitro is entropy-driven. The observed entropy increase here is most likely due to the solvation of salt ions, which have been bound to H1.
before binding to Impβ/Imp7 and, therefore, turns the trimerization into an exergonic process. In contrast to that, it appears likely that the entropic contribution to the interaction in a living cell is much less, since H1 is not soluble in the cytosol in the absence of cytosolic chaperones and, thus, does not bind so many salt ions. Hence, focusing on enthalpy changes possibly reflects the in vivo situation better, with less aberrations.

When taking the binding enthalpy of the heterodimerization of Impβ and Imp7 under identical conditions into account (\(\Delta H_{\text{Impβ-imp7}} = -47\ \text{kJ/mol at 10 }^\circ\text{C}\)), it is obvious that the heterodimerization process compensates in part for the endothermic process of the subsequent H1 binding by Impβ/Imp7 in vitro: \(\Delta H_{\text{Impβ-imp7}} + \Delta H_{\text{Impβ-imp7-H1}} = -47.5\ \text{kJ/mol} + 58.7\ \text{kJ/mol} = -11.2\ \text{kJ/mol}\). It is tempting to confer this result to the in vitro situation, but a direct transfer of the complete heat energy from the Impβ/Imp7 dimerization onto H1 binding is unlikely, whereas an energy transfer by entropy storage is probable.

Consecutively, we investigated by isothermal calorimetry experiments (Fig. 7D) if additional effects account for cooperativity of H1 binding by Impβ/Imp7 besides possibly balancing the energy requirements of substrate binding by dimerization of Impβ and Imp7. Compared with the binding isotherm of Imp7 and H1, the isotherm of an Imp7 fragment lacking IBB, but displaying a complete H1 binding site, Imp7_N-1001, shows that in the absence of IBB, the affinity of Imp7 to H1 does not significantly change. Thus, in contrast to Impα, where an autoinhibitory function of IBBα before binding to Impβ has been reported (22, 23), an autoinhibitory effect of IBB7 on Imp7 can be excluded. In that case one would expect an increase in affinity to H1 when deleting IBB7. When preincubating Imp7_N-1001 with Impβ before titration with H1, the resulting binding isotherm shows no stimulated affinity but, rather, a slightly decreased one, with a \(k_D\) value of 488 nM (Fig. 7B). Hence the IBB7 domain is a prerequisite for cooperativity of Impβ and Imp7 in H1 binding, and this cooperative effect is not simply due to the neutralization of an autoinhibition of Imp7 by Impβ.

Therefore, it appears likely that IBB7 is a kind of messenger of an induced-fit mechanism of one or even both of the receptors in binding H1. This question was addressed by recording binding isotherms of H1 together with one receptor in full-length and the other in a truncated version that is only able to bind the first receptor but not H1. Thus, it is possible to check which receptor is activated by dimerization. In a first step a potential activation of Impβ was examined by titrating a preincubated Impβ/IBB7, complex with H1. IBB7 has already been shown to be defective in binding H1 (Fig. 3B). A stimulating effect of IBB7 on Impβ to H1 cannot be observed; the resulting \(k_D\) of 408 nM (Figs. 7D and 8B) resembles the \(k_D\) of full-length Impβ with H1 (336 nM, Fig. 7C and 8B). Note that the stoichiometry in this experiment turned out to be 3:1 (Impβ:IBB7:H1), indicating a non-physiological mode of interaction. To analyze a possible activation of Imp7 by Impβ, a binding isotherm of Impβ_N-396 and H1 was recorded initially, as a minor part of the Impβ binding site for H1 is still present in this deletion. Although it did not bind H1 in pulldown assays and gel filtration experiments

**FIGURE 6.** The Ran binding site of Impβ mediates the dissociation of the H1 import complex from the NPC. A, in vitro nuclear import assays with subsequent confocal laser scanning microscopy reveal a prominent staining of the nuclear envelope when applying Impβ 127–641-Imp7. DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; Retic, reticulocyte lysate. 8, an immunofluorescence detection of His-tagged proteins in H1 nuclear import shows a nuclear trapping of His-Imp7_598-C. C, a quantification of nuclear-localized immunofluorescence demonstrates that Imp7_598-C accumulates in the nucleus with increasing receptor concentrations. For the noise reduction the value of the immunofluorescence signal in the negative control was subtracted from all other values before the evaluation. The immunofluorescence signal of the full-length heterodimer Impβ-His-Imp7 was set to 1. Receptor concentrations are as indicated.
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A

B

C

D

E

| K_d (nM) | ΔH (kJ/mol) | ΔS (μJ/K mol) | N |
|---------|-------------|---------------|---|
| Impj + IBB7 | 71 ± 7 | -66.9 ± 0.5 | -89.6 | 1.01 ± 0.01 |
| Impj_N-396 + IBB7 | 216 ± 33 | -44.9 ± 1.0 | -23.1 | 0.98 ± 0.02 |
| Impj_N-641 + IBB7 | 40 ± 5 | -52.3 ± 0.6 | -67.4 | 0.97 ± 0.01 |
| Impj_127-C + IBB7 | 256 ± 60 | -58.6 ± 1.7 | -55.1 | 1.02 ± 0.02 |
| Impj_210-C + IBB7 | 1662 ± 641 | -59.4 ± 20.6 | -123.1 | 0.27 ± 0.07 |

| K_d (nM) | ΔH (kJ/mol) | ΔS (μJ/K mol) | N |
|---------|-------------|---------------|---|
| Impj + IBB7 | 71 ± 7 | -66.9 ± 0.6 | -89.6 | 1.01 ± 0.01 |
| Impj/ImprGDPNP + IBB7 | 506 ± 61 | -67.0 ± 1.5 | -177.1 | 0.87 ± 0.02 |

| K_d (nM) | ΔH (kJ/mol) | ΔS (μJ/K mol) | N |
|---------|-------------|---------------|---|
| Imp7 + H1 | 273 ± 20 | 50.0 ± 0.8 | 308.9 | 0.50 ± 0.01 |
| Impj + H1 | 336 ± 77 | 66.7 ± 3.8 | 359.6 | 0.90 ± 0.02 |
| Impj/Imp7 + H1 | 126 ± 13 | 58.7 ± 0.7 | 339.9 | 0.97 ± 0.01 |

| K_d (nM) | ΔH (kJ/mol) | ΔS (μJ/K mol) | N |
|---------|-------------|---------------|---|
| Imp7 + H1 | 273 ± 7 | 50.0 ± 0.6 | 308.9 | 0.50 ± 0.01 |
| Impj/Imp7_N-1001 + H1 | 356 ± 35 | 48.4 ± 1.3 | 294.7 | 0.50 ± 0.01 |
| Impj/Imp7_N-1001 + H1 | 488 ± 66 | 50.9 ± 1.8 | 301.0 | 0.97 ± 0.02 |
| Impj/IBB7 + H1 | 408 ± 49 | 44.1 ± 2.1 | 278.4 | 0.33 ± 0.01 |

| K_d (nM) | ΔH (kJ/mol) | ΔS (μJ/K mol) | N |
|---------|-------------|---------------|---|
| Impj + H1 | 273 ± 7 | 50.0 ± 0.8 | 308.9 | 0.50 ± 0.01 |
| Impj_N-396 + H1 | 658 ± 65 | 79.4 ± 5.1 | 398.9 | 0.25 ± 0.01 |
| Impj_N-396/Imp7 + H1 binding by Impj_N-396 | 1183 ± 105 | 48.3 ± 2.7 | 284.2 | 0.25 ± 0.00 |
| Impj_N-396/Imp7 + H1 binding by imp7 | 166 ± 24 | 34.9 ± 1.0 | 293.3 | 1.00 ± 0.01 |
Imp7 is now significantly increased by Impβ_N-396 with a $k_D$ of 166 nM, in comparison to Imp7 and H1 without Impβ ($k_D = 273$ nM, Figs. 7C and 8B). The binding enthalpy of roughly $-35$ kJ/mol is $\sim$40% lower than that of Impβ/Imp7 and H1 ($\sim59$ kJ/mol) due to only little contributions of Impβ_N-396 to the binding enthalpy. The entropy changes are reduced as well, with $\Delta S = 253.3$ J/K·mol ($\text{Impβ/Imp7} + \text{H1}$: $\Delta S = 339.9$ J/K·mol), indicating less salt ions being released from the surface of H1. Note that the receptor fragment Impβ_N-396 has already been saturated before ($n = 0.25$), and its energetic contribution can be considered low. This is probably also true in the case of a re-ordered binding of Impβ_N-396 to H1, since then the dissociation energy of the initial binding of Impβ_N-396 to H1 has to be taken into account as well. In conclusion, the experiments clearly indicate that the partial storage of energy within Impβ upon heterodimerizing with Imp7 (Fig. 7A) does not directly affect the interaction of Impβ/Imp7 with H1. In fact, the results show that the cooperativity of Impβ/Imp7 is mediated mainly by IBB7, conferring an activation of Imp7 by Impβ.

**DISCUSSION**

In the nuclear import process of the linker histone H1 the heterodimerization of the nuclear import receptors Impβ and Imp7 before H1 binding is an important step upon which both importins act cooperatively in binding their substrate (13). By dissecting the single steps of H1 nuclear import in this study, the IBB7 domain could be identified to be the mediator of cooperativity of Impβ and Imp7 in H1 binding. The entropy decrease on the heterodimerization of Impβ and Imp7 (Fig. 7A) indicates a partial storage of energy within Impβ/IBB7, most likely due to a compression of the superhelical structure of Impβ upon binding to IBB7, but such structural changes have recently been reported for interac-

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**FIGURE 7. Energetic characterization of the trimeric Impβ/Imp7/H1 import complex by ITC.** A, the heterodimerization of Impβ and IBB7 is an exothermic reaction. The N terminus of Impβ is necessary for wild-type level of binding Imp7. Impβ fragments with extending N-terminal deletions, Impβ-127-C and Impβ-210-C, show an increasing loss of affinity to IBB7. A decrease of affinity is also coherent with extended C-terminal deletions, as is demonstrated by Impβ_N-396. Wild-type levels of IBB7 binding are only given with HEAT repeats 1–14 being present (Impβ_N-641). B, upon association with RanGDPNP, Impβ is still able to bind Imp7, yet with significantly reduced affinity. C, binding of H1 to importins is an endothermic reaction. Impβ and Imp7 act cooperatively in binding H1. Upon dimerization the affinity of Impβ/Imp7 to H1 is more than doubled in comparison to the single receptors. Note, whereas the single receptors bind two at a time to H1 ($n = 0.5$), a stoichiometry with $n = 1$ is only achieved with Impβ/Imp7. D, when Imp7_N-1001, lacking IBB7, is titrated with H1 (Impβ_N-1001 + H1), the resulting binding isotherm is similar to full-length Imp7 (Imp7 + H1). Therefore, IBB7 has no significant autoinhibitory effect on Imp7 in the absence of Impβ. In the absence of IBB7, as demonstrated for preincubated Impβ and Imp7_N-1001, which subsequently have been titrated with H1 (Impβ/Imp7_N-1001 + H1), a cooperative effect of both receptors on binding H1 cannot be observed. Notably, the presence of IBB7 has no stimulating effect on Impβ affinity to H1 (Impβ/IBB7 + H1); instead, the stoichiometry of 2:1 ($n = 0.33$) indicates a non-physiological binding (marked by an asterisk). E, the affinity of Imp7 to H1 is stimulated by Impβ. Impβ_N-396 only weakly binds to H1 with a stoichiometry of $n = 0.25$ but significantly enhances the affinity of Imp7 when preincubated with Imp7 before titration with H1. The first binding event represents binding of Impβ_N-396 to H1 with $n = 0.25$ (blue fitting curve), and the second binding event represents the interaction of Imp7 and H1 with $n = 1$ (green curve). The stoichiometry indicates that Imp7 in complex with Impβ_N-396 adopts the same role as when bound to full-length Impβ.

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**FIGURE 8. Summary of binding affinities derived from ITC experiments.** A, binding affinities of full-length Impβ and truncated fragments thereof to IBB7. The affinity of Impβ to IBB7 is set to 100%; the other affinities represent the quotients of the affinity of full-length Impβ and the affinity of the respective mutant. B, binding affinities of Impβ, Imp7, Impβ/Imp7, and of different truncated complexes thereof to H1. The affinity of Impβ/Imp7 to H1 is set to 100%; the other affinities are calculated as described in A.
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tions of Impβ with other binding partners (for review, see Ref. 21). Disregarding the probably artificial entropic effects of the high salt buffer on the interaction of the heterodimer with H1 in the ITC experiments and focusing on the enthalpy changes (Fig. 7, A and C), it becomes evident that the partial storage of energy in the heterodimerization of Impβ and Imp7 in vitro has the capacity to turn the subsequent substrate binding from an enthalpy unfavorable into a more balanced process. Moreover, Imp7 presumably undergos structural changes upon dimerizing with Impβ, allowing an induced-fit mechanism of Imp7 binding to H1. This accounts for the indispensability of an Impβ-Imp7 heterodimer in H1 nuclear import. Interestingly, it is known for members of the Impβ superfamily that cargo binding is often aided by an induced fit. A pronounced structural flexibility of several proteins of this superfamily has already been shown by small angle x-ray scattering experiments (24) and likely is due to the periodical organization of HEAT-tandem repeats within these proteins. Recently, the comparative analysis of three-dimensional structures of HEAT repeat proteins led to the proposal that these proteins in general might make use of their structural flexibility for balancing the energies of complex assembly and disassembly (21). The authors assumed that a distortion of these proteins by an induced-fit-type substrate binding mechanism might store potential energy as in a tightly wound spring. This potential energy might be used for allowing complex association and dissociation by the investment of the relatively small amount of energy provided by RanGTP hydrolysis.

The results presented here offer the first experimental evidence supporting these assumptions, since they indicate the storage of energy in the Impβ-Imp7 heterodimer in the form of an entropy decrease (Fig. 7A). Furthermore, the results also point to a common strategy of β-karyopherins to overcome energetic barriers in binding demanding substrates like H1 by dimerization. In that sense the probable conformational change of Imp7 upon dimerizing with Impβ thermodynamically facilitates an induced-fit mechanism of H1 binding by Imp7. This points toward an allosteric activation of Imp7 by Impβ. When taking the position of the H1 interaction site of Imp7 into account (Fig. 4), which is located at the C terminus (aa 665–1001) but clearly separated from the IBBα domain (aa 1008–1038), this appears even more feasible. Comparing the binding modes of Impβ and known cargoes such as the parathyroid hormone-related protein PTHR-P or Impα, a significant feature of Impβ becomes evident; it is able to force the interaction sites of its binding partners to build up secondary structure elements, which are not present in the Impβ-free state. An N-terminal moiety of the nonclassical nuclear localization signal of PTHR-P forms a β-strand-like conformation (25), whereas the IBB domain of Impα adopts an α-helix when bound to Impβ (8). This feature of IBBα might apply to the IBB domain of Imp7 as well, which would support a conformational change of Imp7 upon dimerization with Impβ. Furthermore the question arises if there are other comparable effects on Impα and Imp7 upon binding to Impβ. However, in contrast to the IBB domain of Impα, which exhibits an autoinhibitory influence on Impα before dimerization with Impβ (22, 23, 26), an autoinhibitory function of IBB7 before binding to Impβ can be excluded, as demonstrated (Figs. 3, 5A, panel 5, and 7D). This finding again emphasizes the exceptional position of IBB7, among other known IBBs and renders it unique. Its biochemical role solely seems to be the mediation of cooperativity of Impβ and Imp7 in H1 binding before import. These observations are supported by the lack of similarities between the IBBα domain and other known IBBs (supplementary Fig. 4). A direct comparison between IBBβ and IBBα reveals a lack of residues in IBBα, that could interact with the acidic loop of Impβ, connecting the A and B helix of HEAT 8 (aa 332–342). IBBα directly interacts with the acidic loop of Impβ with a lysine that is in second position after lysine 18, namely lysine 20. In a comparable position in Impβ, there is no such basic residue present. In fact, in contrast to IBBβ, IBBα displays no charged and only few polar residues beyond lysine 1018, rendering an interaction between the acidic loop of Impβ and IBBα, very unlikely. Rather, it probably is this very feature of IBBα, that allows for a simultaneous binding of both Imp7 and RanGTP to Impβ, yet with a strongly reduced affinity of Impβ to Imp7. This refutes the assumption that binding of Imp7 and RanGTP to Impβ are mutually exclusive (12, 13). However, the difference in entropy changes between the interactions of Impβ and IBBα, and Impβ-RanGDPNP and IBBα, (Fig. 7B) may imply structural changes of the complex between Impβ and IBBα, in the presence of RanGTP in comparison to the interaction in the absence of Ran. This may include an increased rigidity of the whole RanGTP-Impβ-IBBα complex compared with Impβ-IBBα, as well as a rearrangement of the binding site on Impβ for IBBα. Because any interpretation of this phenomenon is currently too speculative, it is likely that only a crystal structure will provide the information needed for an exhaustive explanation.

Remarkably, for complex formation of Impβ and RanGTP, the acidic loop of Impβ has been reported to be crucial (7, 8), pointing to a proverbial “displacement” mechanism for Impα release from Impβ upon RanGTP binding. Our findings cast a similar mechanism in the release of Imp7 from Impβ into doubt but, rather, imply a strictly allosteric regulation of the Impβ-Imp7 interaction by Ran. This is in accordance with recent findings obtained interpreting a crystal structure of full-length Impβ and RanGTP (5). There, an allosteric regulation of Impβ binding to Impα by RanGTP has been postulated. The authors assume a mechanism in which an initial binding of RanGTP to the Impβ C terminus unzips the interaction interface of Impβ and Impα by inducing a conformational change of Impβ which then is followed by a subsequent, competitive displacement of the cargo.

When focusing again on substrate binding by Impβ-Imp7, it becomes obvious that the versatility of the Impβ-Imp7 heterodimer in binding several H1 subtypes points to a general import mechanism for any H1 subtype. Additionally, the involvement of extended acidic loops at the C terminus of Imp7 (aa 882–912 and 927–957) and of the acidic loop of Impβ (turn between helix A and B of HEAT 8, aa 333–343) in H1 binding is a strong argument for Impβ and Imp7 being
not only transporters but potent cytosolic chaperones as well. In the context of chaperoning activity of \textit{Imp}\textbeta{} and \textit{Imp}7, the RanGTP-mediated dissociation of the trimeric \textit{Imp}\textbeta{}-\textit{Imp}7-H1 complex after the passage through the NPC becomes even more momentous.

Because not only \textit{Imp}\textbeta{} but also \textit{Imp}7 can bind RanGTP with high affinity, the question arises whether in the presence of RanGTP an \textit{Imp}7-H1 complex would immediately dissociate at the nuclear basket as well. The reduced affinity of \textit{Imp}7 alone to H1 in comparison with the \textit{Imp}\textbeta{}-\textit{Imp}7 dimer supports such an assumption. In that case none of the importins would serve as a chaperone for H1 in the karyoplasm; other chaperones would then be required to allow a safe passage of linker histones to the nucleosomes. In fact, nuclear chaperones for H1 linker histones do exist (for review, see Ref. 27), and their involvement in completing the delivery of H1 histones to the nucleosomes is likely. Hence, we propose a more detailed model of nuclear import of H1 linker histones including these assumptions (see supplemental Fig. 5).

In summary, the nuclear import of linker histones is allosterically regulated in two major steps of the import process, namely cargo recognition by the \textit{Imp}\textbeta{}-\textit{Imp}7 heterodimer and dissociation of the trimeric \textit{Imp}\textbeta{}-\textit{Imp}7-H1-complex by RanGTP inside the nucleus at the nuclear pore complex. \textit{Imp}\textbeta{} allosterically activates \textit{Imp}7 via its IBB\textsubscript{\textbeta{}} and, this activation is the cause for cooperativity. This suggests that a conformational change of \textit{Imp}7 upon binding to \textit{Imp}\textbeta{} is likely, pointing to an induced fit mechanism of binding H1. Additionally, the dimerization of \textit{Imp}\textbeta{} and \textit{Imp}7 before H1 binding is an energetically favorable process. Because the heterodimerization of both importins is able to compensate for the energy requirements of H1 binding \textit{in vitro}, the necessity of an import receptor dimer for the nuclear import of linker histones is underlined.

\textit{Imp}\textbeta{} turns out to be a general allosteric regulator of co-receptors and adaptors in nuclear import, not only including H1 import, as demonstrated in this study for \textit{Imp}7, but also classical nuclear localization signal import with \textit{Imp}8. Strikingly, \textit{Imp}8 is not always the modulator of co-receptors and adaptors but can also be a target of modulation processes, as was demonstrated for the nuclear import of U1 small nuclear ribonucleoproteins (28). In that case the IBB domain of SPN1 (IBB\textsubscript{SPN1}) modulates \textit{Imp}\textbeta{} in a specific way, which allows for the dissociation of \textit{Imp}\textbeta{} from Nup153 independent of RanGTP, in contrast to the Nup153-\textit{Imp}\textbeta{}-IBB\textsubscript{\textbeta{}} complex. Hence, concerning modulations by or of \textit{Imp}\textbeta{} and the changing energy requirements of different import pathways depending on these modulations, \textit{Imp}\textbeta{} appears to be the center of a cross-talk of nuclear import processes.

Because not only H1 is imported into the nucleus by the \textit{Imp}\textbeta{}-\textit{Imp}7 heterodimer but also the integrase of human immunodeficiency virus-1 (HIV-1-IN) (29), in this case with \textit{Imp}7 being the protagonist and \textit{Imp}\textbeta{} only a minor player, it will be of major importance to obtain crystal structures of H1 and HIV-1-IN in complex with \textit{Imp}\textbeta{}-\textit{Imp}7 to understand how in detail \textit{Imp}7 is regulated by \textit{Imp}\textbeta{} and how HIV-1-IN adopts the features of cellular cargoes such as H1 to make use of a very similar import pathway as well.

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REFERENCES
1. Madrid, A. S., and Weis, K. (2006) Chromosoma (Berl.) \textbf{115}, 98–109
2. Pemberton, L. F., and Paschal, B. M. (2005) Traffic \textbf{6}, 187–198
3. Fried, H., and Kutay, U. (2003) Cell. Mol. Life Sci. \textbf{60}, 1659–1688
4. Conti, E. (2002) Results Probl. Cell Differ. \textbf{35}, 93–113
5. Lee, S. J., Matsuura, Y., Liu, S. M., and Stewart, M. (2005) Nature \textbf{435}, 693–696
6. Matsuura, Y., and Stewart, M. (2004) Nature \textbf{432}, 872–877
7. Choock, Y. M., and Blobel, G. (1999) Nature \textbf{399}, 220–237
8. Cingolani, G., Petosa, C., Weis, K., and Müller, C. W. (1999) Nature \textbf{399}, 221–229
9. Görlich, D., Dabrowski, M., Bischoff, F. R., Kutay, U., Bork, P., Hartmann, E., Prehn, S., and Izaurralde, E. (1997) \textit{J. Cell Biol.} \textbf{138}, 65–80
10. Jäkel, S., and Görlich, D. (1998) \textit{EMBO J.} \textbf{17}, 4491–4502
11. Lam, M. H. C., Briggs, L. J., Hu, W., Martin, J., Gillespie, M. T., and Jans, D. A. (1999) \textit{J. Biol. Chem.} \textbf{274}, 7391–7396
12. Jäkel, S., Albig, W., Kutay, U., Bischoff, F. R., Schwamborn, K., Doenecke, D., and Görlich, D. (1999) \textit{EMBO J.} \textbf{18}, 2411–2423
13. Bäuerle, M., Doenecke, D., and Albig, W. (2002) \textit{J. Biol. Chem.} \textbf{277}, 32480–32489
14. Kutay, U., Izaurralde, E., Bischoff, F. R., Mattaj, I. W., and Görlich, D. (1997) \textit{EMBO J.} \textbf{16}, 1153–1163
15. Bedenken, J., Cingolani, G., and Gerace, L. (2003) \textit{J. Cell Biol.} \textbf{162}, 391–401
16. Melchior, F., Sweet, D. J., and Gerace, L. (1995) \textit{Methods Enzymol.} \textbf{257}, 279–291
17. John, J., Frech, M., and Wittinghofer, A. (1988) \textit{J. Biol. Chem.} \textbf{263}, 11792–11799
18. Adam, S. A., Marr, R., and Gerace, L. (1990) \textit{J. Cell Biol.} \textbf{111}, 807–816
19. Jäkel, S., Mingot, J. M., Schwarzsmaier, P., Hartmann, E., and Görlich, D. (2002) \textit{EMBO J.} \textbf{21}, 377–386
20. Görlich, D., Pante, N., Kutay, U., Aebi, U., and Bischoff, F. R. (1996) \textit{EMBO J.} \textbf{15}, 5584–5594
21. Conti, E., Müller, C. W., and Stewart, M. (2006) \textit{Curr. Opin. Struct. Biol.} \textbf{16}, 237–244
22. Harren, M. T., Hodel, M. R., Fanara, P., Hodel, A. E., and Corbett, A. H. (2003) \textit{J. Biol. Chem.} \textbf{278}, 5854–5863
23. Harren, M. T., Cohen, P. E., Hodel, M. R., Truscott, G. J., Corbett, A. H., and Hodel, A. E. (2003) \textit{J. Biol. Chem.} \textbf{278}, 21361–21369
24. Fukushima, N., Fernandez, E., Ebert, J., Conti, E., and Svergun, D. (2004) \textit{J. Biol. Chem.} \textbf{279}, 2176–2181
25. Cingolani, G., Bedenken, J., Gillespie, M. T., and Gerace, L. (2002) \textit{Mol. Cell} \textbf{10}, 1345–1353
26. Kobe, B. (1999) \textit{Nat. Struct. Biol.} \textbf{6}, 388–397
27. Tyler, J. K. (2002) \textit{Eur. J. Biochem.} \textbf{269}, 2268–2274
28. Huber, J., Dickmanns, A., and Lührmann, R. (2002) \textit{J. Cell Biol.} \textbf{156}, 467–479
29. Fassati, A., Görlich, D., Harrison, I., Zaytseva, L., and Mingot, J. M. (2003) \textit{EMBO J.} \textbf{22}, 3675–3685