Distinct importin recognition properties of histones and chromatin assembly factors

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Received 9 September 1999; received in revised form 24 December 1999

Edited by Masayuki Miyasaka

Abstract Synthesis of the protein components of nuclear chromatin occurs in the cytoplasm, necessitating specific import into the nucleus. Here, we report the binding affinities of the nuclear localisation sequence (NLS)-binding importin subunits for a range of histones and chromatin assembly factors. The results suggest that import of histones to the nucleus may be mediated predominantly by importin β1, whereas the import of the other components probably relies on the conventional α/β1 import pathway. Differences in recognition by importin β1 were observed between histone H2A and the variant H2AZ, as well as between histone H3/4 with or without acetylation. The results imply that different histone variants may possess distinct nuclear import properties, with acetylation possibly playing an inhibitory role through NLS masking.

Key words: Importin; Histone; Histone variant; Chromatin-associated protein; Histone acetylation; NAP-1

1. Introduction

Approximately 2 m of DNA is condensed into a compartment 20 μm in diameter in eukaryotes through a hierarchical scheme of folding and compaction into a protein-DNA assembly called chromatin. The nucleosome is the regularly repeated basic unit of chromatin and consists of approximately 146 bp of superhelical DNA wrapped twice around a core of two of each of four histones H2A, H2B, H3 and H4. Interaction of the H1 class of histones with the DNA between nucleosomes enables higher order compaction (for review, see [1]). The transfer of histones onto DNA involves various histone-binding proteins, such as nucleoplasmin [2,3], N1/N2 [2] and NAP-1 [4,5]. Nucleoplasmin and NAP-1 are associated with H2A and H2B transfer onto DNA, whereas N1/N2 is associated with H3 and H4 in *Xenopus* oocytes [3]. Ordered disassembly of chromatin is vital in gene transcription, with acetylated histones and histone variants often present in regions of chromatin with higher transcriptional activity. In terms of non-histones, the chromatin-associated high mobility group (HMG) HMG-14 and -17 proteins bind the nucleosome directly in such a way as to alter nucleosome structure and potentially subsequent chromatin folding, and thus facilitate remodelling to a more transcriptionally active structure [5–7].

Histones are synthesised in the cytoplasm and thus must subsequently be translocated to the nucleus. The absolute and extensive requirement for histones during DNA replication implies the necessity for specific mechanisms of histone nuclear import. Protein import into the nucleus is generally mediated by specific targeting signals called nuclear localisation sequences (NLSs) [8]. Conventionally, NLS-containing proteins are recognised specifically by importin α [9], which is in a heterodimeric association with importin β, followed by docking of the whole complex to the nuclear pore through importin β [10,11]. After translocation through the pore mediated by the monomeric guanine nucleotide-binding protein Ran [12], the NLS-containing proteins and importin α are released from importin β into the nucleoplasm. More recent studies have shown that the nuclear import of certain proteins can be mediated exclusively through importin β, independent of importin α [13–15].

The archetypal NLS is that of the SV40 large tumour antigen (T-ag) [8], comprising a short stretch of basic amino acids ($PKKKRKV^{132}$). Bipartite NLSs, in contrast, contain two short stretches of basic amino acids separated by a 10–12 amino acid spacer. Transport of proteins harbouring either T-ag-like or bipartite NLSs is mediated through importin α/β, with NLS-binding performed directly by importin α [16,17]. Sequence motifs similar to the T-ag NLS have been identified in histones H1 [18] and H2B [19], whereas N1/N2, HMG-14 and -17 appear to contain bipartite NLSs [16,20] (Table 2).

Very little is known with respect to the transport of histones and chromatin assembly factors into the nucleus. As a first step towards understanding this process, we used an enzyme-linked immunosorbent assay (ELISA)-based assay [21,22] to determine the affinities of recognition of histones, nucleosome-associated proteins and chromatin assembly factors by importin α, β and the α/β heterodimer. The results indicate that translocation of histones to the nucleus is most likely mediated through pathway(s) dependent on importin β but independent of importin α, whereas translocation of chromatin-associated proteins is probably through the conventional importin α/β-mediated pathway.

2. Materials and methods

2.1. Protein expression and purification

2.1.1. Histones and chromatin-associated proteins. Histone H1 was

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PII: S0014-5793(00)01142-X

from Boehringer Mannheim (Cat. No. 223549). Histones H2A and H2B from *Xenopus laevis* and H2AZ from mouse were expressed from the vector pET3a in *Escherichia coli* strain BL21DE3lysS and prepared as described from inclusion bodies [23]. Briefly, 2 ml of histone raw extract was incubated overnight in 50 ml of 50 mM HEPES (pH 7.5) containing 6 M urea, and subsequently passaged through a 0.22 μm filter before loading onto a Resource S FPLC column, pre-equilibrated with 50 mM HEPES (pH 7.5) containing 6 M urea, at 0.5 ml/min. Fractions were collected every 0.5 min in a 0–2 M NaCl gradient in 50 mM HEPES (pH 7.5) containing 6 M urea, and histone-containing fractions identified by gel electrophoresis and silver staining. Histones were subsequently refolded and dimerised as described [23]. Histones H3/4 and acetylated H3/4 (H3/4-ac) were purified from chicken and HeLa cells, respectively, as previously described [24]. Recombinant human HMG-14 and -17 were expressed and purified as described [25].

2.1.2. Recombinant NAP-1 and N1/N2. Plasmid N1/N2 106/3 containing the N1/N2 coding region was kindly donated by Juergen Kleinschmidt [26]. The N1/N2 coding region was ligated into pQE2 (Qiagen), to generate plasmid pQE-N1/N2. The T7-tagged yeast NAP-1 coding sequence was subcloned from plasmid pTN2, a kind gift from Fugii Nakata [4], filled-in and blunt-end-ligated into *Sma*I-digested pQE30 (Qiagen) to generate plasmid pQE-NAP-1.

N1/N2 and NAP-1 were expressed in *E. coli* and denatured using 8 M urea before purification by Ni-NTA column chromatography using a method modified from Thanos and Maniatis [27]. Briefly M15(rep4) cells were grown to an $OD_{600\,nm}$ of 0.5–0.7 and then induced with 1 mM IPTG for 4 h at 37°C. The cell pellet was collected by centrifugation and resuspended in 20 ml buffer A (8 M urea, 100 mM sodium phosphate, pH 8, 10 mM Tris–HCl, pH 8, 10 mM imidazole, 0.5 mM PMSF, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol) and incubated for 20 min at room temperature. Cellular debris was removed by centrifugation at 10 000 rpm at room temperature and the supernatant loaded onto a 1 ml Ni-NTA agarose (Qiagen) column equilibrated with buffer A containing 20 mM imidazole. The column was washed with 20 ml 20 mM imidazole buffer A and the proteins eluted with buffer A containing 100 mM imidazole. Fractions (300 μl) were analysed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and peak fractions pooled and renatured by dialysis against 6 M urea, 500 mM sodium chloride, 20 mM HEPES, pH 7.9, 10% (v/v) glycerol, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, 0.1% NP-40, with urea removed in step-wise fashion over 72 h. Insoluble material was removed by centrifugation at 14 000 rpm at 4°C, and the proteins dialysed against EB storage buffer (1 mM EGTA, 20 mM HEPES, pH 7.5, 1.5 mM magnesium chloride, 10% (v/v) glycerol, 50 mM DTT, 10 mM 2-mercaptoethanol, 60 mM potassium chloride).

2.1.3. Importin-glutathione-S-transferase (GST) fusion proteins. Importin α2 (PTAC58) and β1 (PTAC97) subunits from mouse were expressed as GST fusion proteins and purified by glutathione affinity chromatography, as previously [21,22]. All samples were stored in small aliquots at −70°C until use.

2.2. ELISA-based binding assay

Binding affinities of importins for the various proteins were quantitated using an ELISA-based assay [16,17,21,22]. Peptide P101, comprising T-ag amino acids 111–132 including the NLS, was used as a conventional NLS control [28,29]. Proteins and peptides were coated onto microtiter plates and then incubated with increasing concentrations of importin subunits or the pre-hybridised importin α/β heterodimer. Importin-binding was then measured using a GST-specific primary antibody (Amersham Pharmacia) and a secondary antibody (Sigma Immunochemicals) conjugated to alkaline phosphatase. The colourimetric reaction after addition of *p*-nitrophenyl phosphate substrate (Sigma) was followed at 405 nm for 90 min on a microtiter plate reader (Molecular Devices). Values were corrected against background absorbance at 0 min and against wells incubated without importin.

3. Results

3.1. Histones are recognised with high affinity by importin β

An ELISA-based assay, previously used to characterise the importin-binding properties of a number of NLS-containing proteins that localise to the nucleus [21,22,30], was used to determine the binding constants for histones. Peptide P101 [28,29] containing the T-ag NLS was used as a positive control, showing high affinity binding by importin α/β (apparent dissociation constant, K_D, of 3.1 ± 0.3 nM, $n = 7$) [21,22]. This binding was predominantly through the α subunit (K_D of 50 nM, $n = 3$, compared to a K_D for the importin β subunit of 202 nM, $n = 2$; see also [16,17]). Although all histones were recognised with high affinity by importin α/β, this appeared to be predominantly through importin β, since maximal binding by importin β was 85–100% that by importin α/β (Figs. 1 and 2; Table 1), and the binding by importin α was low in all cases ($K_D > 50$ nM). H1 showed the highest binding affinity for importin β of 2.7 ± 0.2 nM (Table 1 and Fig. 1); further underlining the fact that importin α/β-binding to H1 was predominantly through importin β was the lack of a significant difference in the binding affinity of importin β in either the absence or presence of importin α (Student's *t*-test, $P = 0.1$).

Histones H2A and H2B are believed to be transported to the nucleus as heterodimers [19]. We compared the differences in importin recognition between H2A/2B, and H2AZ/2B which contains the H2A variant H2AZ [31] (Fig. 2). Importin α, β and α/β all showed markedly better binding to H2AZ/2B than to H2A/2B ($P = 0.009$ for importin α/β, Table 1 and Fig. 2A), as indicated by both the lower K_Ds, and the > 50% higher maximal binding by importin α/β and β to H2AZ/2B. As for histone H1, the binding by importin α/β appeared to be predominantly through importin β (Fig. 2A and Table 1). We also compared recognition by importin subunits of H3/4 without or with pre-acetylation (Fig. 2B). The affinity of importins α/β and β for H3/4 was significantly diminished ($P = 0.01$ and 0.0009, respectively) when H3/4 was acetylated, as was the maximal binding (by > 60%), but binding remained predominantly associated with the β rather than the α subunit (Table 1 and Fig. 2B).

3.2. Importin-binding properties of chromatin-associated HMG-14 and -17 proteins

The ELISA assay was also used to assess importin-binding

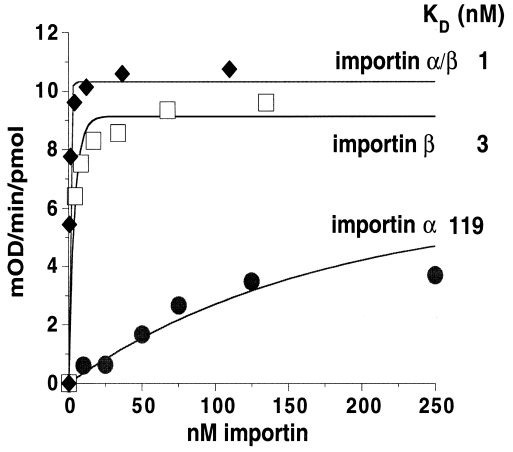

Fig. 1. Histone H1 is recognised with higher affinity by importin β than importin α, as determined using an ELISA-based assay. Data were fitted for the function $B(x) = B_{max}(1-e^{-kt})$, where x is the concentration of importin and B is the level of importin bound. The apparent dissociation constants (K_D), representing the concentration of importin at which the level of binding is half-maximal, are indicated. Results shown are from a single typical experiment, performed in triplicate, with pooled data shown in Table 1.

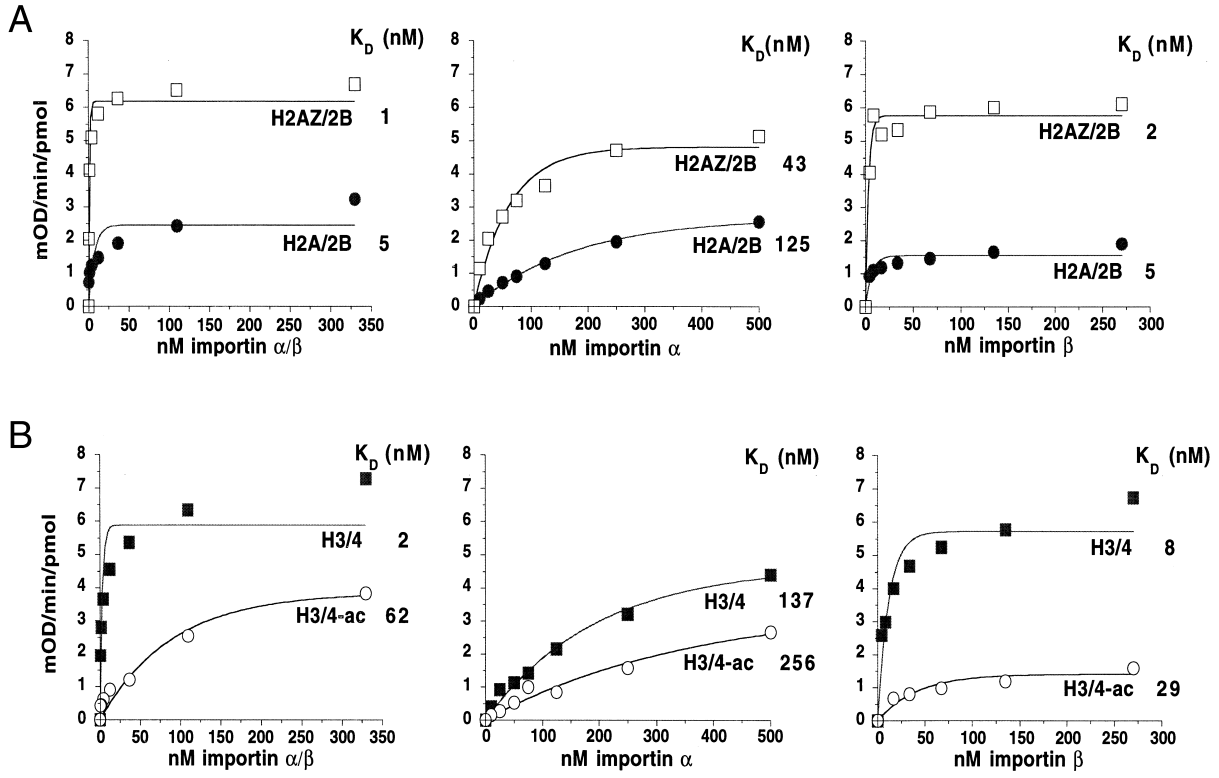

Fig. 2. Importin β binds histones with high affinity. Experimental data were generated as described in the legend to Fig. 1. Results for importin α/β, α and β are shown in the left, middle and right panels, respectively, for histones H2A/2B and H2AZ/2B (A), and non-acetylated and acetylated histones H3/4 (B).

to HMG-14 and -17. Importin α mediated recognition of HMG-14 and -17 by the importin α/β heterodimer as indicated by the fact that maximal binding by importin α was markedly greater than that by importin β alone (Table 1 and Fig. 3A). HMG-14 and -17 were recognised with similar affinity by the importins (Fig. 3A), which may stem from the high sequence conservation within the proposed NLS motifs of these proteins [20].

3.3. Importin-binding properties of chromatin assembly factors

The affinity of importin-binding to the chromatin assembly factors N1/N2 and NAP-1 was similarly assessed. Importin α/β showed high binding affinity for N1/N2 (K_D of 1.6 nM similar to that of P101 containing the T-ag NLS, see Section 3.1) [17,21], with importin α-binding marginally more strongly than importin β (Table 1 and Fig. 3B). In contrast, NAP-1 failed to be recognised by either importin α/β (K_D of about 115) or the importin α or β subunits individually (Table 1 and Fig. 3B).

4. Discussion

This study presents quantitative results for recognition by importin α/β of histones and other chromatin-associated nu-

Table 1
Binding affinities of importins for histones and chromatin-associated proteins

Protein	Importin α/β		Importin α			Importin β		
	K_D (nM)[a]	B^a_{max}	K_D (nM)	B_{max}	α/β (%)[b]	K_D (nM)	B_{max}	α/β (%)[b]
H1	1.4 ± 0.6 (3)	10.7 ± 0.2	96.3 ± 11.4 (3)	5.2 ± 0.5	48	2.7 ± 0.2 (3)	10.6 ± 1.0	99
H2A/2B	9.2 ± 3.6 (4)	3.8 ± 0.8	207.2 ± 82.2 (3)	4.2 ± 1.2	109	5.5 ± 0.5 (3)	3.3 ± 1.3	87
H2AZ/2B	1.1 ± 0.4 (3)	8.0 ± 1.0	56.1 ± 13.1 (2)	5.7 ± 0.9	71	4.0 ± 1.6 (3)	8.9 ± 1.6	111
H3/4	3.7 ± 1.4 (3)	6.3 ± 0.3	152.7 ± 26.7 (3)	1.5 ± 1.0	72	8.2 ± 0.1 (3)	5.4 ± 0.3	86
H3/4-ac[c]	56.6 ± 10.4 (4)	2.6 ± 0.5	252.6 ± 43.6 (3)	3.5 ± 1.1	134	29.0 ± 3.4 (3)	1.1 ± 0.1	41
HMG-14	15.5 ± 4.0 (3)	8.8 ± 2.2	200.5 ± 6.1 (3)	3.1 ± 0.8	35	173.8 ± 38.0 (4)	2.5 ± 0.6	29
HMG-17	3.5 ± 1.2 (4)	9.7 ± 1.6	187.0 ± 29.4 (3)	4.2 ± 0.7	43	149.0 ± 2.0 (3)	2.5 ± 0.8	26
N1/N2	1.6 ± 0.1 (3)	10.7 ± 3.1	150.4 ± 32.5 (3)	2.9 ± 0.6	27	197.2 ± 31.4 (3)	3.9 ± 1.3	23
NAP-1	115.0 ± 29.0 (3)	1.5 ± 0.3	248.0 ± 23.4 (4)	2.8 ± 0.3	183	89.4 ± 8.7 (3)	1.0 ± 0.4	68

[a]Binding parameters were determined using an ELISA-based assay (see Figs. 1–3), as described in Section 2 [21,22]. Curves were fitted to experimental data using the function $B(x) = B_{max}(1-e^{-kt})$, where x is the concentration of importin and B is the level of importin bound. K_D is the apparent dissociation constant for the interaction between importin and the protein in question, and represents the importin concentration at which binding is half-maximal. B_{max}, expressed in mOD/min/pmol, is the maximum level of importin-binding. Results represent the mean ± S.E.M. Individual experiments were performed in triplicate, with the number of experiments indicated in parentheses.
[b]The B_{max} of the respective individual importin subunit is expressed as a percentage of that for α/β.
[c]Acetylated histone H3/4.

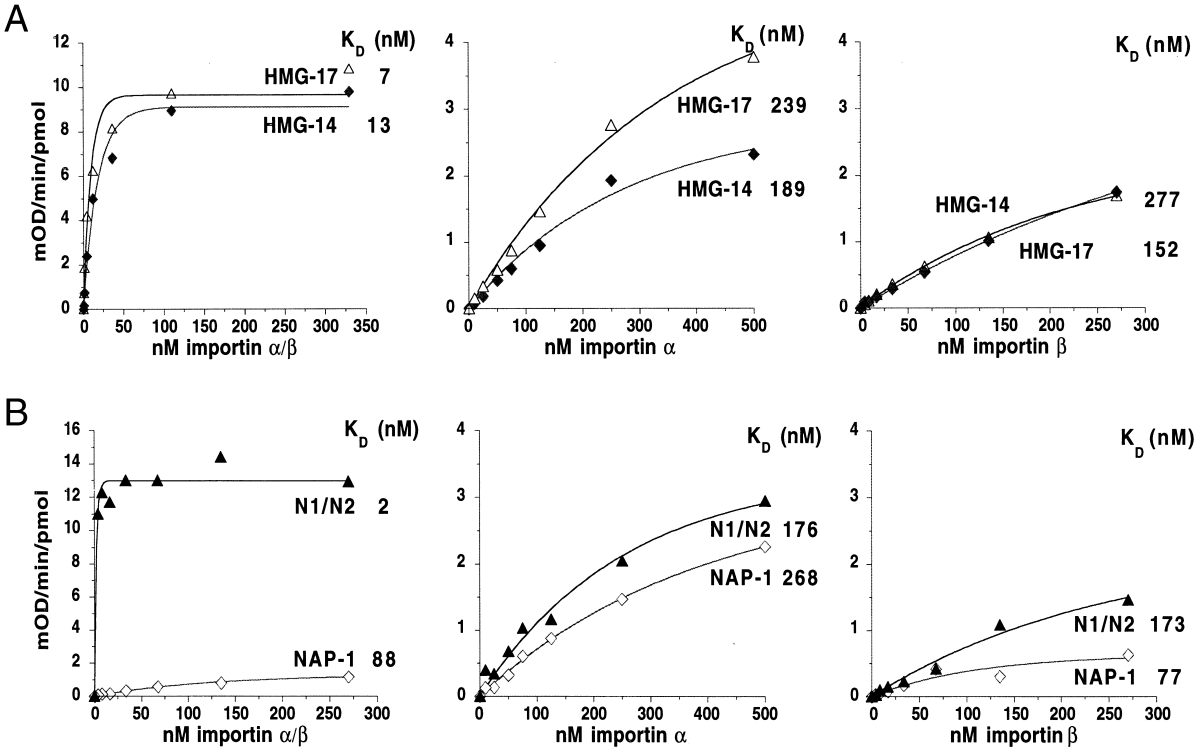

Fig. 3. Importin α binds the chromatin-associated proteins with higher affinity. Experimental data were generated as described in the legend to Fig. 1. Results for importin α/β, α and β are shown in the left, middle and right panels, respectively, for HMG-14 and -17 (A), and N1/N2 and NAP-1 (B). It should be noted that the scale of the *y*-axis is higher for the right-most panels.

clear proteins, and chromatin assembly factors. Binding of the importin α/β heterodimer was consistently high for all of the proteins, with the exception of NAP-1 which was not recognised with high affinity by importin α, β or α/β. Histones were predominantly recognised by the importin β subunit, whereas the chromatin assembly factors were recognised by importin α/β, predominantly through the action of importin α.

Where analysed, histone nuclear import has been shown to be dependent on cytosolic factors, but inhibited by wheat germ agglutinin and non-hydrolysable GTP analogues, indicating dependence on components of the nuclear pore and Ran [18,32]. Despite multiple sequence elements in H1 resembling the T-ag NLS [18] (Table 2) and clearly being able to be recognised by importin α/β (see Table 1) [18,32], H1 import into the nucleus has been reported to be effected by importin β in a heterodimeric association with importin 7, based in part on the observation that nuclear import can be reconstituted in vitro using human importin β1 and *Xenopus* importin 7 [32]. Consistent with this, we show in this study that there is a high affinity (nM range) interaction between importin β and histone H1, of 50-fold higher affinity than that between importin α and histone H1. Our study and that of Jaekel et al. [32] thus imply that importin α is unlikely to play a role in H1 nuclear uptake, in contrast to conventional NLS-containing proteins such as T-ag where importin α mediates NLS recognition. Several recent reports have implicated importin β as the sole mediator of nuclear import of HIV-1 Rev [13,34], HTLV-1 Rex [35] and GAL4 [14]. These proteins have in common with histones the fact that they all bind either DNA or RNA, implying that high affinity of importin may be an emerging feature of a group of proteins that may be broadly classified as DNA/RNA-binding. Other proteins such as cyclin B1 [36] and PTHrP [15] which appear not to bind DNA or RNA, however, also appear to be imported into the nucleus by this pathway.

Although nuclear protein import pathways mediated by importin β1 alone, or as a heterodimer with importin 7 [32], have not been delineated in detail, it is clear that the affinities for histone recognition by importin β1 determined here (3–8 nM, see Table 1) are within the range of those of other published importin β1 import substrates such as PTHrP (ca. 2 nM) [15] and GAL4 (ca. 12 nM) [14]. The physiological relevance of these affinities can be judged in the context of the affinity of RanGTP for importin β1 (0.8 nM) [37], and the fact that the cellular concentrations of importin β1 and Ran (e.g. ca. 3 and 10 μM, respectively, in *Xenopus* oocytes [38–40]) are well in excess of the binding/dissociation affinities measured here and elsewhere [14,15,32,37]. That the cellular concentration of total importin β family members (ca. 20 μM) in *Xenopus* oocytes is higher than that for Ran [38] with even lower concentrations in HeLa cells [39] means that the apparent high intrinsic affinity of histone-binding by importins may be critical in competition for Ran [40]; there would also appear to be intense demand for importin β1, not only by proteins recognised by heterodimers in which importin β1 is involved such as histone H1 variants, and of course conventional NLS-containing proteins, but also by directly bound import substrates such as PTHrP or TCPTP.

H2AZ differs from H2A at various residues along the entire protein with differential extensions at the C-terminus [41]. These differences are believed to account for disruptions in the integrity of the interaction between the H2A/H2B dimer and H3/H4 tetramer in the nucleosome core, and play a critical functional role in *Drosophila melanogaster* [42], *Tetrahy-*

Table 2
Putative and confirmed basic NLS sequences and acetylation sites in histones and other chromatin-associated proteins

Protein	Basic Sequence*	NLS Confirmed
H1°	**PVKKAKKKLAATPKKAKK**^{159} **KTVKAKPVKASKPKKAKPVKPKAKSSAKRAGKKK**^{193}	Sufficient for nuclear localisation [18].
H2B	⇓ ⇓ ⇓ ⇓ KSAPAPKKGSKKAATKTQ**KKGDKKRM**^{30}	Sufficient for nuclear localisation [19].
H3	⇓ ⇓ ⇓ ⇓ ⇓ KQTARKSTGGKAPRKQLATK^{23}.....**KKPHRYR**^{42}	Predicted
H4	⇓ ⇓ ⇓ ⇓ SGRGKGGKGLGKGGA**KRHRK**^{20}	Predicted
HMG-14	**PKRK**^{4}.....KPKK^{32}.....**KGKRG**^{54}	Necessary for nuclear localisation [20].
HMG-17	**PKRK**^{4}.....PKKKAPAKK^{48}.....**PKGKKG**^{58}	Necessary for nuclear localisation [20].
N1/N2	**RKKRK**TEEESPLKD**KAKKSK**^{554}	Necessary and sufficient for nuclear localisation [16].

*The NLSs are highlighted in bold. Acetylation sites, occurring at lysine residues in the histone N-termini close to the putative NLSs, are indicated by vertical arrows [33].

mena thermophila [43] and mouse development [31]. No conventional NLS-like motif has been identified in H2A or H2AZ. Since these histones appear to be transported to the nucleus as heterodimers with H2B [19], high affinity recognition by importin α/β and also possibly importin β can probably be attributed to the H2B NLS (GKKRSKA^{33}, see Table 2); nuclear import of H2B, however, can occur in the absence of this sequence [19], possibly through an additional importin-independent pathway (see below). We hypothesise that the H2B NLS may be rendered partly inaccessible when H2B is associated with H2A, but due to the different nature of the binding by H2AZ, may be more readily recognised by importins. Consistent with this hypothesis, the region of H2B recognised by H2A (residues 37–114 in calf thymus-derived histones [44], and residues 40–119 in yeast) is close to the H2B NLS, meaning that a masking effect may account for our observation that H2AZ/2B is recognised with a higher affinity than H2A/2B by importin α/β and importin β. Distinct histone variants may thus be transported to the nucleus with differing efficiency, meaning that the transport of certain histones may be favoured under particular conditions such as during development, which is indeed the case for H2AZ in *Drosophila* [42]; the latter may also relate to the fact that whereas H2AZ is synthesised throughout the cell cycle, major histones such as H2A are only made at S-phase.

Histone acetylation is believed to play a role in the transcriptional permissiveness of chromatin [33]. Acetylation of lysine residues on the N-terminal tails of H3 and H4 (Table 2) neutralises their positive charge, thereby decreasing the affinity of their interaction with DNA and facilitating decompaction and disassembly of chromatin to increase accessibility to transcription factors. The region of acetylation in H3 contains NLS-like sequences (Table 2), and we observed here that importin α/β and β recognised H3/4-ac with significantly lower affinity than non-H3/4-ac. The fact that acetylation in H4 occurs at K^{16} close to the putative NLS implies that it may exert a masking effect, with the other acetylated lysine residues in the N-termini of H3 and H4 (see Table 2) possibly playing a similar role in masking the importin-binding site of H3 or H4. Since histone acetylation clearly occurs in the cytoplasm [45], the clear implication of this study is that acetylated histones, including H3 and H4, may be transported to the nucleus with only poor efficiency through importin β.

Although they also play a crucial role in nucleosome structure, chromatin assembly factors differ from histones in that they do not bind DNA directly, but rather interact with the histones. HMG-14 and HMG-17 harbour bipartite NLSs (Table 2) and importin has been shown to be involved in their nuclear uptake, which interestingly appears to be regulated according to the stage of the cell cycle [20]. Our results here for the HMG proteins also indicate interaction primarily through importin α, in similar fashion to other bipartite NLS-containing proteins [16,17,46]. Similarly, the importin recognition properties of N1/N2, which also has a functional bipartite NLS (Table 2), were consistent with this. Unlike the other chromatin assembly factors, importin α/β-binding was negligible for NAP-1. NAP-1 has no motif with any consensus for an NLS [47], but clearly localises to the nucleus during S-phase [48] and may well play a role as a shuttle protein translocating H2A/2B into the nucleus, in addition to its chromatin assembly role [5]. This nuclear histone import function of NAP-1 would thus appear to be independent of importin α/β; whether NAP-1 is recognised by one of the newly identified importin β homologs [49,50] remains to be seen.

In conclusion, our results indicate that the import of histones into the nucleus is likely to be mediated by importin β independently of importin α, whereas that of histone-binding chromatin-associated proteins and N1/N2 may rely on the more conventional importin α/β pathway. Histones thus appear to be transported to the nucleus by a pathway [32] distinct from that of other chromatin components and chromatin assembly factors. The advantage to the cell would appear to be that this provides the possibility of regulating the nuclear import of these classes of proteins in a different fashion, according to requirements during the cell cycle, development etc. [20,42].

Acknowledgements: We thank Luke Hyman for skilled technical assistance, and Mark Lam for critical reading of the manuscript.

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