

# Cloning of two novel human importin- $\alpha$ subunits and analysis of the expression pattern of the importin- $\alpha$ protein family

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**Abstract** The import of many proteins into the nucleus is mediated by the importin- $\alpha/\beta$  heterodimer. While only one importin- $\beta$  gene has been found, several forms of importin- $\alpha$  have been described. In addition to the three human importin- $\alpha$ s already identified, we report here the primary structure of two new human importin- $\alpha$  proteins. The five known human importin- $\alpha$  subunits can be classified into three subfamilies that appear conserved in higher eukaryotic organisms. We show by immunoblotting that the different importin- $\alpha$  subfamilies are expressed in a variety of human tissues and mammalian cell lines.

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**Key words:** Nuclear transport; Importin- $\alpha$ ; Protein expression; Nuclear signaling

## 1. Introduction

The transport of proteins from the cytoplasm into the nucleus through the nuclear pore complex (NPC) [1] is an energy-dependent process [2,3] that is triggered by specific signals (for review see [4]). The best characterized import signal is the 'classical' nuclear localization signal (NLS) that consists of one or more clusters of basic amino acids [5]. Four soluble factors are known to be involved in the NLS-dependent nuclear import: importin- $\alpha$  [6–9], importin- $\beta$  [10–13], the GTPase Ran/TC4 [14,15], and pp15 [16,17]. Importin has also been termed 'karyopherin'.

Importin- $\alpha$  and importin- $\beta$  form a heterodimeric complex in the cytoplasm. Complex formation depends on the presence of the importin- $\beta$  binding (IBB) domain, which is located in the amino-terminal part of the  $\alpha$ -subunit [18,19]. During protein transport, the importin complex binds to the NLS of the import substrate in the cytoplasm. [11,20]. The NLS recognition complex docks to the NPC and is subsequently translocated through the pore by an energy requiring, Ran-dependent mechanism [2,3,15]. At the nucleoplasmic side of the NPC, the three components separate and importin- $\alpha$  and the substrate reach the nucleoplasm [21,22]. Finally, importin- $\alpha$  and importin- $\beta$  are re-exported from the nucleus to the cytoplasm [18,19].

Both importin- $\beta$  and importin- $\alpha$  have been identified in all eukaryotic organisms examined thus far. While *SRP1* [23] is

the only gene that encodes an importin- $\alpha$  homologue in *Saccharomyces cerevisiae*, other organisms possess several importin- $\alpha$  genes, e.g. hSRP1 [24], Rch1 [25], and Qip1 [26] in human and homologues of Rch1 and hSRP1 in mouse [27]. In contrast, only one gene for importin- $\beta$  is found in a given organism. In vitro analysis using a cell-free import system [28] demonstrated that both Rch1 and hSRP1 support the import of NLS-bearing substrates [22]. However, in vitro binding studies also indicated that proteins with different NLS may have different affinities for various importin- $\alpha$  forms [26].

Here we present the sequences of two novel human importin- $\alpha$  proteins. Sequence comparison suggests that the importin- $\alpha$  subunits can be classified into three subfamilies, namely a SRP1-like subfamily, a Rch1-like subfamily, and an importin- $\alpha$ 3/Qip1-like subfamily. Analyzing the distribution of human forms, we found that all but importin- $\alpha$ 6 are expressed ubiquitously. However, the molar ratio of the distinct importin- $\alpha$  proteins varies in different tissues and cell types. Moreover, peptide-specific antibodies also recognize the importin- $\alpha$ 3-like proteins in cells from other mammals, indicating that these forms are evolutionarily conserved.

## 2. Materials and methods

### 2.1. Standard techniques

Database screening, multiple alignment, and sequence analysis were essentially performed as described in [11]. cDNA clones were sequenced on both strands. Colony hybridization, PCR, and DNA sequencing were done as described [7] with modifications indicated in the text. Raising of antibodies, affinity purification, and immunoblotting were performed as described in [29].

### 2.2. Isolation of novel importin- $\alpha$ cDNAs

A partial importin- $\alpha$ 3 cDNA-clone (accession number H02648) was obtained from the Reference Library ICRF and labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham) by the Rapid Multiprime DNA Labeling Kit (Amersham) to screen a HeLa pUEX cDNA library. One positive clone was isolated of about 1.3 kbp length, which harbored the proposed start codon of importin- $\alpha$ 3. The 3' end of the cDNA was cloned using the HeLa Marathon-Ready cDNA RACE kit (Clontech) and the primers TCCTCTGCTCAGCCACCAGGGAG and GTGG-AAGGAAAGATCAAGTGG according to the manufacturer's instructions. Two independent positive clones were isolated and sequenced. Full length cDNA was obtained by PCR using primers ATATCCATGGCGGACAACGAGAAA and ATATGGATCCAACTGGAACCCCTTCT. Importin- $\alpha$ 4 was isolated from a human lung library (Clontech) during a two-hybrid system screen. For isolation of importin- $\alpha$ 6, 5' RACE and 3' RACE were performed with a HeLa Marathon-Ready cDNA kit and the primers TTCTGCTTAGATTCTTGTCTCCA and ATAGTCCAAGTGGCTTTAAATGA respectively, which correspond to a human cDNA clone (accession number AA043363). Products were cloned and sequenced. Several positive clones, which harbored the proposed start codon or the proposed stop codon respectively, were identified. Full length

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**Abbreviations:** NLS, nuclear localization signal; NPC, nuclear pore complex; IBB, importin- $\beta$  binding domain; arm, armadillo repeat; ORF, open reading frame

cDNA was isolated using primers ATATGCATGCATGCCATGGC-TAGT and ATATGGATCCAAGTTGAAATCCATCCA.

2.3. Northern blotting

Human multiple tissue Northern blots (Clontech) were hybridized according to the manufacturer's instructions with an [ $\alpha$ -<sup>32</sup>P]dCTP-labeled 0.6 kbp cDNA fragment from importin- $\alpha$ 3. The blots were washed and re-probed with a 1.0 kbp fragment of importin- $\alpha$ 4, with a 1.3 kbp fragment of importin- $\alpha$ 6, and with a 0.35 kbp fragment of GAPDH cDNA.

2.4. Cell culture and isolation of total protein

HeLa, HepG2 (human liver carcinoma cells), 293 (embryonic kidney cells), and MRC-5 (human lung fibroblasts) were cultured at 37°C in 5% CO<sub>2</sub> with Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal calf serum (Sigma), and gentamicin. Jurkat (human T cells), Raji (human B cells), T23/96 (human glioma cells) were cultured in RPMI 1650 medium (Gibco) supplemented with 10% fetal calf serum, and gentamicin. ECV 304 were grown in Medium 199 Earle (Seromed) supplemented with 10% fetal calf serum, penicillin/streptomycin, and protection medium (Seromed). Human umbilical vein endothelial cells (HUVEC) were cultured in Medium 199 Earle supplemented with 10% fetal calf serum, and penicillin/streptomycin. When subconfluent, cells were washed twice with ice cold PBS (Seromed) and harvested by scraping in 10 ml ice cold PBS. After centrifugation at 1300 rpm for 10 minutes at 4°C, pellets were resuspended in 300 ml lysis buffer (50 mM Tris-HCl pH 7.3, 2% SDS, 1 mM PMSF) and sonicated. After centrifugation for 5 min at 13000 rpm, lysates were incubated for 5 min at 95°C and stored at -20°C until use. Protein concentrations were measured with the Micro BCA Protein Assay Reagent Kit (Pierce).

2.5. Generation of antibodies and immunoblotting

Antibodies were raised in rabbits against peptide sequences of the amino-termini of the different human importin- $\alpha$  proteins: MSTNE-NANTPAARLC (Rch1), MTPGKGFENRFLC (hSRP1), MAD-NEKLDNQRLC (importin- $\alpha$ 3), MAENPSLENHRIC (importin- $\alpha$ 4). Cell protein lysates (15  $\mu$ g per lane) and human protein medleys (25  $\mu$ g per lane) (Clontech) were separated using 10% SDS-PAGE. Detection was achieved by chemiluminescence (Du Pont).

3. Results and discussion

3.1. Cloning of novel importin- $\alpha$  proteins

When we began our study, only two human importin- $\alpha$  proteins were known, hSRP1 and Rch1. By screening the GenBank database, we identified three different classes of partial human cDNA sequences, which displayed partial homologies with both hSRP1 and Rch1. We set out to isolate the corresponding full length cDNA clones (see Section 2). The cDNAs represented three new proteins: importin- $\alpha$ 3, importin- $\alpha$ 4, and importin- $\alpha$ 6. While our work was in progress, Seki et al. [26] reported the sequence of importin- $\alpha$ 3 as Qip1. The deduced protein sequences of all three cDNAs showed the main characteristics of importin- $\alpha$ : the amino-terminal IBB domain, the eight armadillo (arm) repeats, and a carboxy-terminal acidic region (Fig. 1). Therefore, these proteins probably perform similar functions during protein import into the nucleus.

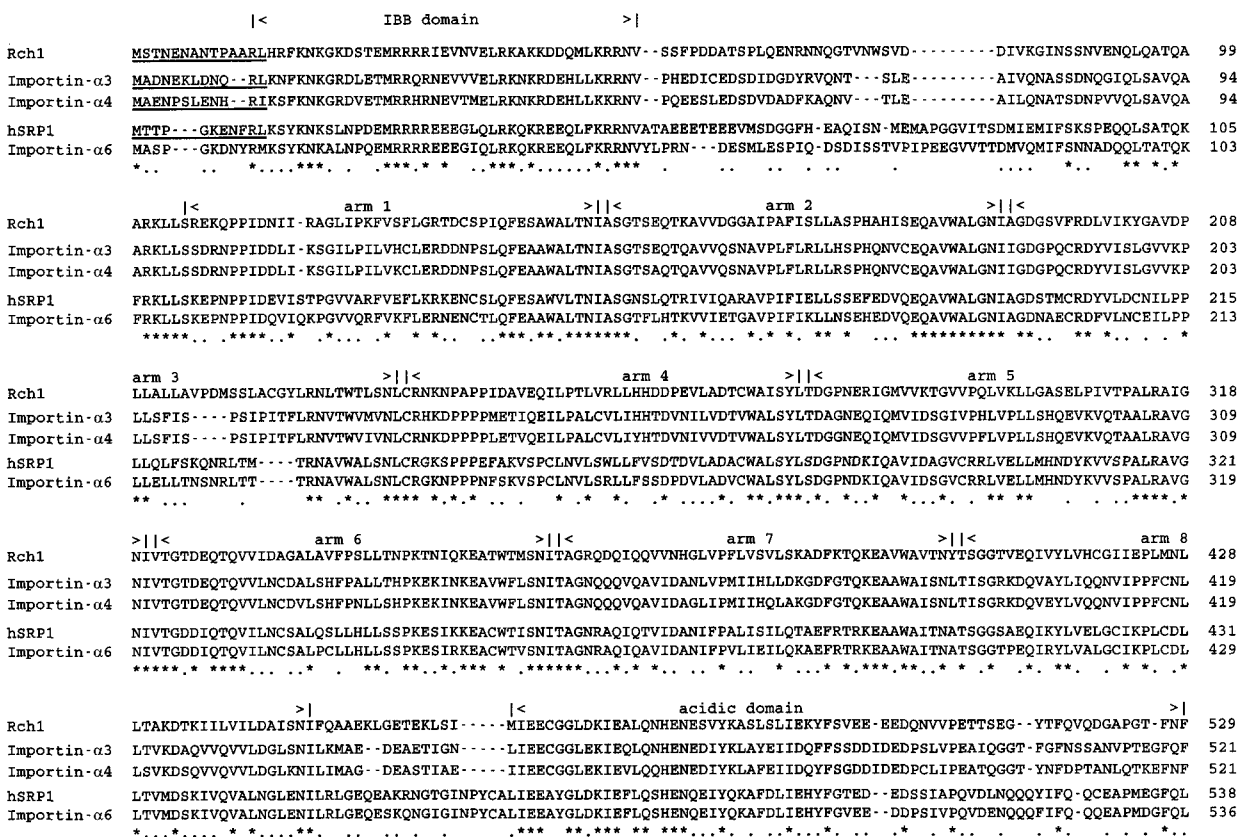


Fig. 1. Multiple alignment of the human  $\alpha$ -subunits of importin. Protein sequences were aligned using the CLUSTAL program. Identical residues are indicated with a star, similar residues with a dot. Epitopes used for antibody raising are underlined. Protein domains are indicated in the upper line. IBB domain, importin- $\beta$  binding domain; arm, armadillo repeat. GenBank accession numbers of the new importin- $\alpha$ s are: U93240 and Y12393 (importin- $\alpha$ 3), Y12394 (importin- $\alpha$ 4), AF005361 (importin- $\alpha$ 6).

### 3.2. The novel importin- $\alpha$ proteins belong to different subfamilies

Based on the similarity of their primary structures, the five known human importin- $\alpha$  proteins belong to three subfamilies: a Rch1-like subfamily with Rch1 as the only member, an importin- $\alpha$ 3/Qip1-like subfamily consisting of importin- $\alpha$ 3 and importin- $\alpha$ 4, and a SRP1-like subfamily consisting of hSRP1 and importin- $\alpha$ 6. The protein sequence identity between members of a particular importin- $\alpha$  subfamily is about 80%. The identity between members of different subfamilies is about 50%, and the overall identity between the five proteins is about 33%. Two regions are more divergent, namely the region between the IBB domain and the first arm motif, and the region which links the last arm motif with the carboxy-terminal part. Whether these differences reflect specific functions of the different proteins remains to be clarified. Other importin- $\alpha$  genes may exist in human. For example, GenBank harbors partial cDNA sequences (accession numbers H11822 and T08580) that are most similar but not identical to importin- $\alpha$ 6.

We next compared the human  $\alpha$ -subunits with homologues of importin- $\alpha$  from other species (Fig. 2). We included only complete protein sequences of the non-redundant NCBI protein database with more than 20% identity to hSRP1, which possess the amino-terminal IBB domain [18,19]. Most of the sequences found belong to one of the three subfamilies. The SRP1 subfamily consists of mammalian and yeast SRP1, importin- $\alpha$ 6, and two open reading frames (ORFs) of the plant *Arabidopsis thaliana*. The importin- $\alpha$ 3/Qip1 subfamily includes, in addition to the human members, an uncharacterized ORF from *Caenorhabditis elegans*. Members of the Rch1 subfamily are mammalian pendulin/Rch1, OHO31 from *Drosophila melanogaster*, and the two importin- $\alpha$  proteins that have been isolated from *Xenopus laevis* eggs. These data suggest that the three human subfamilies of importin- $\alpha$  are conserved throughout evolution. In addition, two groups of partial cDNA sequences from *D. melanogaster* exist, which encode importin- $\alpha$  proteins belonging to the SRP1 subfamily and the importin- $\alpha$ 3/Qip1 subfamily, respectively (data not shown). This finding indicates that invertebrates may also possess importin- $\alpha$  subunits of all three subfamilies simultaneously. In contrast, two of the uncharacterized ORFs in *C. elegans*

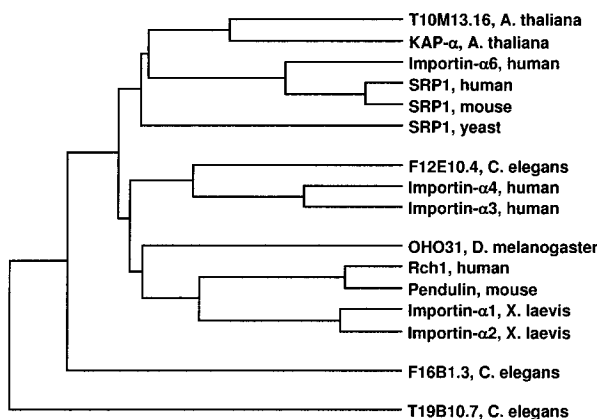


Fig. 2. Alignment tree of all known importin- $\alpha$  homologues. Tree construction was performed using the CUSTAL program. The accession numbers of the uncharacterized ORFs are: AF001308 (T10M13.16), U41992 (F12E10.4), U80444 (F16B1.3), Z74043 (T19B10.7).

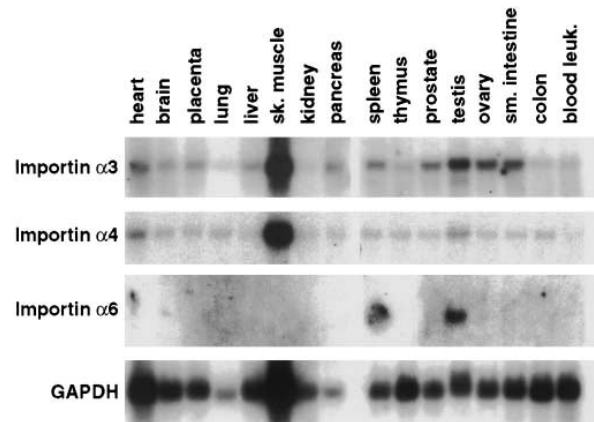


Fig. 3. Novel importin- $\alpha$  mRNAs are differentially expressed in human tissues. Two human multiple tissue Northern blots were hybridized with probes specific for importin- $\alpha$ 3, importin- $\alpha$ 4, importin- $\alpha$ 6, and GAPDH as control. sk. muscle, skeletal muscle; sm. intestine, small intestine; blood leuk., peripheral blood leukocytes.

clearly do not belong to one of the three subfamilies (Fig. 2). Possibly, these ORFs have hitherto unidentified members in vertebrates as well. However, whether these proteins are involved in nuclear import or whether they have other functions is unknown.

### 3.3. Distribution of the mRNA of novel importin- $\alpha$ subunits in human tissues

We performed RNA analysis to determine the tissue distribution of the three novel importin- $\alpha$  subunits. Transcripts of 4.4 kbp (importin- $\alpha$ 3) and 4.6 kbp (importin- $\alpha$ 4) were detected in human poly(A)<sup>+</sup> RNA from all tissues tested (Fig. 3; see Section 2). However, RNA levels varied considerably between tissues. Importin- $\alpha$ 3 was highly expressed in testis, ovary, small intestine, and pancreas, but barely detectable in kidney, thymus, colon, and peripheral blood leukocytes. The stronger signals in heart and skeletal muscle and the weaker signals observed in lung may be due to differences in the overall amount of mRNA loaded (compare to GAPDH control). Importin- $\alpha$ 4 was found to be minimally expressed in liver, kidney, and peripheral blood leukocytes, but showed higher expression in testis and colon. Again, the levels for importin- $\alpha$ 4 in heart and skeletal muscle correlated with GAPDH hybridization. In contrast, transcripts of importin- $\alpha$ 6 (2.4 kbp) were detected only in testis.

### 3.4. Generation of antibodies recognizing different importin- $\alpha$ proteins

To study the expression of the different importin- $\alpha$  proteins, we raised antibodies against synthetic peptides representing the amino-termini of the subunits. Antibodies which specifically recognize four human importin- $\alpha$  proteins (importin- $\alpha$ 3, importin- $\alpha$ 4, hSRP1, Rch1) were obtained. Cross-reactivity was excluded by hybridization with the different peptides, and with recombinant proteins (Rch1, importin- $\alpha$ 3), and by peptide inhibition studies (data not shown).

### 3.5. Expression of the different importin- $\alpha$ proteins in mammalian cells

We first analyzed total protein lysate from different human

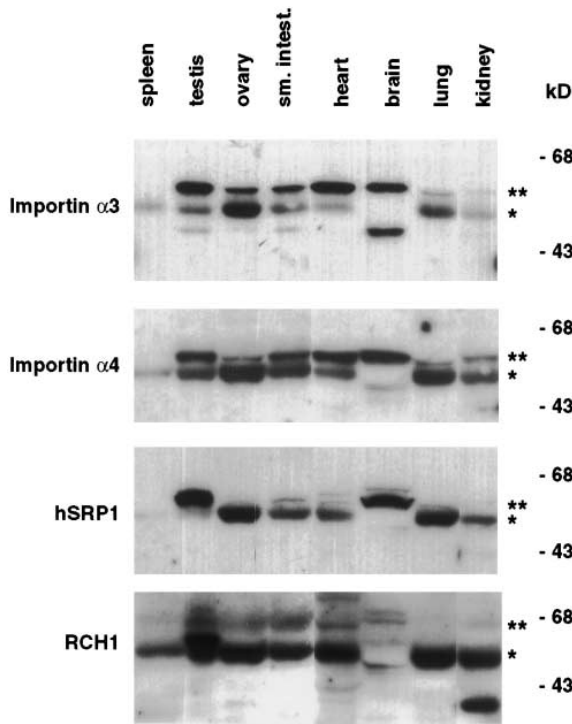


Fig. 4. Importin- $\alpha$  forms are differentially expressed in human tissues at protein level. 25  $\mu$ g of each protein medley was loaded per lane, separated by SDS-PAGE, and probed by immunoblotting using the antibodies raised against the amino-terminal peptides of importin- $\alpha$ 3, importin- $\alpha$ 4, hSRP1, and Rch1 and then visualized by chemiluminescence. \* and \*\* indicate two different migrating bands found for every subunit. sm. intestine, small intestine.

tissues by immunoblotting (see Section 2) (Fig. 4). All antibodies against the importin- $\alpha$  subunits recognized proteins with an expected molecular weight of about 50–60 kDa. Surprisingly, all proteins migrated as double bands (see \* and \*\* in Fig. 4). A similar pattern of bands after human cellular protein immunoblotting of cytosolic and nuclear fractions

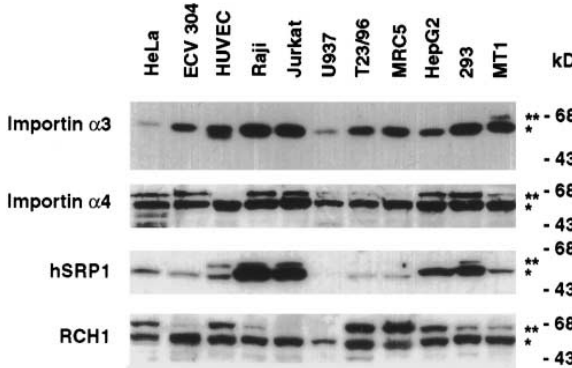


Fig. 5. Importin- $\alpha$  forms are differentially expressed in human cells at protein level. Total cellular proteins from different human cell lines were prepared as described in Section 2. 15  $\mu$ g of protein extracts was loaded per lane and analyzed as described in Fig. 4. \* and \*\* indicate the two different migrating bands found for every subunit. ECV 304, endothelial cells; HUVEC, human umbilical vein endothelial cells; Raji, B cells; Jurkat, T cells; U937, macrophages; T23/96, glioma cells; MRC5, lung fibroblasts; HepG2, liver carcinoma cells; 293, embryonic kidney cells; MT1, mammary tumor cells.

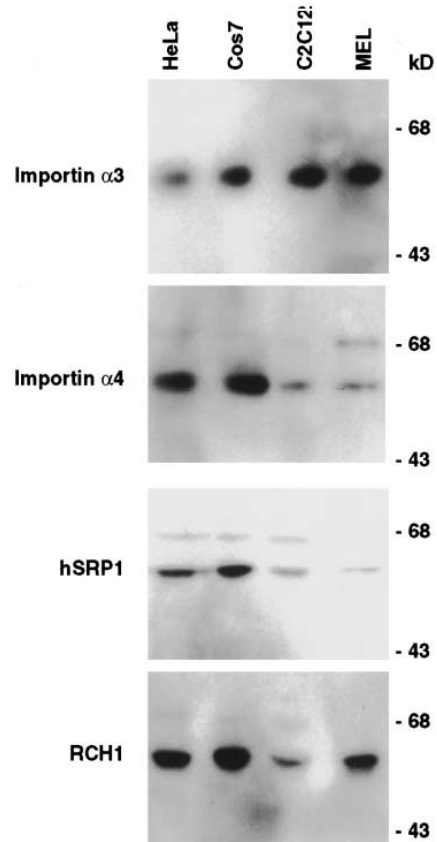


Fig. 6. Expression of importin- $\alpha$ 3 and importin- $\alpha$ 4 in different mammalian cells. Total cellular proteins of cell lines from different mammals were prepared as described in Section 2. 15  $\mu$ g of protein extracts was loaded per lane and analyzed as described in Fig. 4. As a control, total cellular protein from human HeLa cells was loaded as well. Cos7, monkey cells; C2C12, mouse myoblasts; MEL, mouse erythroleukemia cells.

was observed by Nadler et al. [30] using both anti-hSRP1 and anti-Rch1 antibodies. Both bands identified by the peptide-specific anti-Rch1 antibody were also identified with an anti-Rch1 antibody that was raised against the entire protein [11] (not shown). Therefore, we believe that the two immunoreactive bands correspond to closely related proteins, either to post-translationally modified versions of the particular importin- $\alpha$  proteins, or to products of alternatively spliced mRNAs.

Although the diverse importin- $\alpha$  proteins were present in all samples tested, their relative expressions differed. Importin- $\alpha$ 3 was less abundant in spleen and kidney. Rch1 and hSRP1 were expressed weakly in brain and spleen. These data are consistent with the mRNA expression pattern in mouse tissues [27] showing higher levels of mouse pendulin/Rch1 mRNA expression in heart and spleen but a very low level of expression in brain.

Furthermore, we determined the expression of the importin- $\alpha$  subunit in various human cells. Equal amounts of total protein were analyzed by immunoblotting using the four antibodies described in Fig. 4. We detected all importin- $\alpha$  forms in all cell types tested (Fig. 5). However, the total amount of importin- $\alpha$ , as well as the relative content of the particular importin- $\alpha$  forms, varied between the different cell types. Importin- $\alpha$ 4 was more abundant in U937, while in other cells like Raji, Jurkat, 293, or HepG2, hSRP1 is enriched. More-

over, the relative content of importin- $\alpha$ 3 is particularly low in HeLa and in U937.

To examine the phylogenetic conservation of the different importin- $\alpha$  subunits, we tested their expression in green monkey cells (Cos7) and mouse cells (C2C12, MEL) by immunoblotting. We found that not only Rch1 and hSRP1, but also importin- $\alpha$ 3 and importin- $\alpha$ 4 are expressed in green monkey cells, as well as in mouse cells (Fig. 6). This finding indicates that the presence of these proteins is not restricted to human. Because there is also a partial mouse cDNA in the database encoding a protein almost identical to human importin- $\alpha$ 6 (accession number AA438018), we assume that all known human importin- $\alpha$  forms are conserved throughout the mammalian species.

The presence of four different importin- $\alpha$  proteins in probably different modified forms in mammalian cells suggests several NLS-dependent protein import pathways co-existing within one cell type. The significance of the various importin- $\alpha$  subunit expression patterns is currently not known. Possibly, specific importin- $\alpha$  expression patterns are involved in the modulation of signal transduction processes. The importin- $\alpha$  expression level can change dramatically during development [31] or in response to extracellular agents [30]. However, no data exist demonstrating differences in the regulation of expression levels between the various forms. The structural differences between the importin- $\alpha$  forms suggest that they may preferentially import distinct proteins into the nucleus. This assumption is supported by recent data indicating differences in the affinity of Rch1, importin- $\alpha$ 3/Qip1, and human SRP1 to the NLS of DNA helicase Q1 using a two-hybrid assay [26]. However, direct determination of both the affinity of the various importin- $\alpha$  forms for different NLS and the ability of the distinct importin- $\alpha$  forms to support nuclear protein import of various substrates should clarify this matter. Alternatively, importin- $\alpha$  may also be involved in processes other than the import of nuclear proteins; the existence of the different forms may be linked to other functions.

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