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ORIGINAL ARTICLE

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Localization of importin α (Rch1) at the plasma membrane and subcellular redistribution during lymphocyte activation

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Abstract Rch1 belongs to the importin α subfamily and works as an adapter between karyophilic proteins and the nuclear import machinery. Its level of expression varies among species and tissues, and depends on the state of cellular metabolism. In the present study we examined the level of expression of nuclear envelope and nuclear transport proteins (Rch1, importin β, lamins A/C, lamin B, gp210, p62 and transportin) after human lymphocyte activation with phytohemagglutinin. We observed that the level of Rch1 increases dramatically, especially in larger lymphocytes, in response to activation. Moreover, using immunoelectron microscopy, this nuclear transport factor was found to be localized at the plasma membrane and also in tracks from the cytoplasm through the nuclear envelope into the nucleus. Similar localization was also observed in the human melanoma cell line A375. In addition, metabolic activation led to a redistribution of Rch1 from the cytoplasm to both the plasma membrane and the nuclear interior. These results suggest that, during lymphocyte activation, Rch1 may be involved in a signal transduction pathway that involves the shuttling of karyophilic proteins from the plasma membrane to the nucleus.

Introduction

Lymphocyte activation in response to extracellular signals leads to changes in the expression pattern of genes implicated in growth and differentiation (Diehn et al. 2002). The transduction of these signals usually involves the translocation of karyophilic proteins from the cytoplasm to the nucleus. Once inside the nucleus, they bind DNA to regulate the transcription of specific genes (Genot and Cantrell 2000). Modulation of the intracellular localization of karyophilic proteins is usually achieved by post-translational modifications that mask or expose regions of the protein implicated in their localization or DNA binding activity (Kaffman and O'Shea 1999; Jans et al. 2000). However, little is known about the actual physical pathways along which karyophilic proteins travel from their origin in the cytoplasm to their final nuclear destination.

It is evident that the nuclear transport machinery has to be precisely coupled to the aforementioned sequence of events. The first import step is to recognize the activated nuclear localization sequence (NLS) of the karyophilic protein in the cytosol, a process generally mediated by NLS receptors of the importin α family (Görlich et al. 1994; Moroianu et al. 1995a; Weis et al. 1995). Afterwards, the karyophilic protein/importin α complex interacts with importin β (Chi et al. 1995; Görlich et al. 1995; Moroianu et al. 1995b; Radu et al. 1995) to travel across the nuclear envelope and liberate the karyophile in the nucleus with the help of Ran-GTP (Moore and Blobel 1993; Moroianu and Blobel 1995a, 1995b; Görlich et al. 1996; Moore 2001). Several soluble factors participate in the recycling of this transport system (Görlich and Kutay 1999). Regulation can be achieved at multiple steps along this chain, and the presence (Nadler et al. 1997; Sekimoto et al. 1997; Jäkel et al. 1999; Köhler et al. 1999; Welch et al. 1999) or absence (Palacios et al. 1997; Tiganis et al. 1997; Palmeri and Malim 1999; Schedlich et al. 2000) of specific adaptors of the importin α family constitutes a powerful way to control import during its initial stages.

Several isoforms of importin α have been described, and their levels of expression vary among different tissues (Prieve et al. 1996; Köhler et al. 1997; Kamei et al. 1999; Geles and Adam 2001; Giarrè et al. 2002), during embryonic development (Máthé et al. 2000; Lorenzen et al. 2001) and according to the metabolic state of the cell (Nadler et al. 1997; Sekimoto et al. 1997; for a review see Jans et al. 2000). The subcellular distribution of various importin α isoforms has already been described in different species. The importin α family member

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Oho31/pendulin is present at high levels in early *Drosophila* embryos and is rapidly degraded at the end of embryogenesis (Küssel and Frasch 1995; Török et al. 1995). This protein showed cell cycle-dependent nuclear localization, accumulating in the nucleus at the prophase of embryonic cellular divisions. Mutations in Oho31/pendulin led to abnormal development of hematopoietic cells and the central nervous system (Küssel and Frasch 1995; Török et al. 1995). Similarly, Rch1 interacts with Pax5, a transcription factor essential for the development of B cells and the central nervous system (Kovac et al. 2000), while importin α3 was found to be necessary for proper germ line and larval development in *Caenorhabditis elegans* (Geles and Adam 2001).

In the present study, we analyzed the levels of expression of the nuclear transport components Rch1, importin β and transportin, the nuclear envelope components lamin A/C and lamin B, and the nuclear pore proteins gp210 and p62, and determined their subcellular localization during activation of human blood lymphocytes with phytohemagglutinin (PHA). The levels of the NLS receptor Rch1 increased dramatically within 48 h of activation, compared with the other nuclear transport factors analyzed. In addition, by means of immunoelectron microscopy, we detected a pool of Rch1 associated with the plasma membrane that doubled during the activation process. As has been observed with several karyophilic proteins (Meier and Blobel 1992; Kimura et al. 2000; Andrade et al. 2001), Rch1 was also observed in linear tracks from the cytoplasm to the nuclear interior, indicative of its possible association with the cytoskeleton. Overall, our results suggest an important role for Rch1 in the transport of karyophilic proteins from the plasma membrane to the nucleus.

Materials and methods

Antibodies and reagents

Mouse monoclonal antibodies recognizing Rch1, karyopherin β, p62 and transportin were purchased from Transduction Laboratories (catalog nos. R43020, K48020 and N43620, respectively). Rat monoclonal antibody 2G7 against importin α Rch1 was a kind gift from F.A. Grässer (Fischer et al. 1997). Monoclonal anti-lamin B was from Oncogene Sciences (catalog no. NA12) and a polyclonal antibody against lamins A and C was a kind gift from B. Maro (Houliston et al. 1988). gp210 was detected with serum from a patient with primary biliary cirrhosis. Anti-mouse and anti-rat antibodies conjugated with 10 nm gold were purchased from British Biocell International and anti-mouse, anti-human and anti-rabbit antibodies conjugated with fluorescein were obtained from Sigma. Human melanoma cell line A375 was obtained from the American Type Culture Collection and cultured in DMEM supplemented with 10% fetal calf serum, glutamine and antibiotics in an incubator at 37°C and 5% CO_2 atmosphere.

Lymphocyte culture and PHA treatment

Human peripheral blood cells were obtained from healthy donors. Lymphocytes were isolated by means of a Ficoll-Paque PLUS gradient (Pharmacia Biotech), seeded at 2×10^6 cells/ml in RPMI medium and stimulated with 20 μg/ml PHA (Boehringer Mannheim) for 0, 6, 20, 28, and 48 h intervals.

Immunoblotting

Equal numbers of cells from each lymphocyte treatment were loaded per lane and proteins were separated by SDS-polyacrylamide gel electrophoresis in 10% gels and electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell). Blots were blocked with 10% non-fat milk powder in TBST (10 mM TRIS-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 2 h at room temperature. Primary antibodies were incubated overnight at 4°C in 5% milk in TBST at the following concentrations: Rch1 and p62, 50 ng/ml; transportin and karyopherin β, 0.5 μg/ml; lamin A/C, 1:1000; gp210, 1:640; and lamin B, 1:500. Detection of the primary antibody was performed using anti-human, anti-rabbit (Sigma), anti-mouse or anti-rat (Pierce) horseradish peroxidase-conjugated immunoglobulins in TBST, 5% non-fat milk powder followed by Enhanced ChemiLuminescence (ECL) detection (Amersham Pharmacia Biotech).

Immunofluorescence

Lymphocytes were washed in PBS, fixed in 4% formaldehyde and spun onto poly-lysine coated glass slides using a cytorotor in a Megafuge 1.0 centrifuge (Heraeus Sepatech). A375 cells were grown in chamber slides and fixed in 3% formaldehyde. Cells were permeabilized in methanol at −20°C for 5 min and washed in PBS. After blocking with 10% fetal calf serum (Biochrom, KG) in PBS for 1 h at room temperature, cells were incubated overnight at 4°C with anti-Rch1 (2.5 μg/ml in blocking solution). Anti-mouse-fluorescein isothiocyanate (Sigma) was used as a secondary antibody and samples were examined with a 100× oil immersion objective in a Leica Leitz DMRB microscope.

Flow cytometry analysis

Samples were processed as for immunofluorescence, but all the washing and antibody incubation procedures were carried out with cells in suspension. The levels of Rch1 were quantified in an EPICS ELITE flow cytometer (Coulter Electronics, Florida, USA). Mean fluorescence and the coefficient of variation were expressed in arbitrary units on a logarithmic scale. 2×10^4 single-cell events were analyzed in each experiment. List mode files were analyzed with WinMDI 2.8 software, and populations of small and large cells were obtained from the forward angle light scatter versus side scatter biparametric analysis. The results shown are representative of three independent experiments.

Immunoelectron microscopy

Lymphocytes or A375 cells were fixed in 0.5% glutaraldehyde, 4% formaldehyde, 0.1 M cacodylate buffer, pH 7.4 for 4 h at room temperature, washed in iso-osmolar cacodylate/sucrose buffer and incubated in 50 mM NH_4Cl at 0°C for 1 h. After repeated washing, samples were dehydrated through an ethanol series and embedded in Lowicryl K4M as described elsewhere (Renau-Piqueras et al. 1989; Arlucea et al. 1998). Ultrathin sections were deposited on Formvar-coated nickel grids. For post-embedding immuno-microscopy studies, nonspecific binding was blocked with 10% normal goat serum in PBST (0.1% Tween 20 in PBS, pH 8.2). Incubation with monoclonal antibodies against Rch1 (2.5 μg/ml for commercial antibodies and a 1:20 dilution for 2G7 rat antibodies) was performed for 2 h at room temperature in PBST containing 1% normal goat serum and 1% BSA. After five washes in blocking solution, 10 nm gold-conjugated anti-mouse or anti-rat antibodies (British BioCell International) were used at a 1:10 dilution in the same solution as primary antibodies. Finally, grids were washed

five times in blocking solution and distilled water, and stained with uranyl acetate and lead citrate. Control experiments, in which primary antibodies were omitted from the immunocytochemical procedure, did not give rise to labeling. Samples were visualized and electrographed in a 208 S Philips electron microscope at 80 kV.

Quantitative analysis after immunogold labeling

Gold particles in resting and 48 h PHA-activated lymphocytes were counted from electron micrographs of randomly chosen cells for each treatment. The distribution of labeling was determined for the following cellular subregions: (1) cytoplasmic compartment, which was in turn subdivided into areas containing plasma membrane and cytosol; (2) nuclear envelope, which included gold particles restricted to both sides of the nuclear pore complexes, outer and inner nuclear membranes and nuclear lamina; and (3) nuclear interior. For plasma membrane or nuclear envelope gold association, we only included particles that appeared to be in intimate physical contact with each compartment. Statistical analysis for differences in the distribution of gold particles was determined using the χ^2 test.

Results

We detected considerably different levels of protein expression for several nuclear envelope components as well as soluble factors of the nuclear transport machinery during activation of human lymphocytes with PHA. By immunoblotting we observed that the levels of lamin B and transportin remained nearly constant after 48 h of activation, while a moderate increase in the nuclear pore proteins gp210 and p62 and the transport receptor importin β (karyopherin β) could be detected (Fig. 1). On the other hand, there was a pronounced increase in the levels of lamins A and C between 20 and 28 h of activation. Moreover, the expression levels of the importin α homolog Rch1 rose dramatically during the process of activation, from nearly undetectable levels in resting lymphocytes to intense levels following 48 h of PHA exposure (Fig. 1). We further verified this result by quantifying the levels of Rch1 in resting and activated lymphocytes by means of flow cytometry. We found that 48 h PHA-treated cells had nearly three orders of magnitude more Rch1 than the control untreated lymphocytes (Fig. 2). Furthermore, in both treated and untreated cells, two populations could be differentiated according to the size of the constituent cells (i.e., small and large cells, Fig. 2). Thus, in resting lymphocytes, both small and large cells contained nearly the same amount of Rch1. In contrast, in PHA-treated lymphocytes, the proportion of large Rch1-expressing cells increased significantly as a result of activation (Fig. 2, middle), and the level of Rch1 for this population of larger cells reached its highest value.

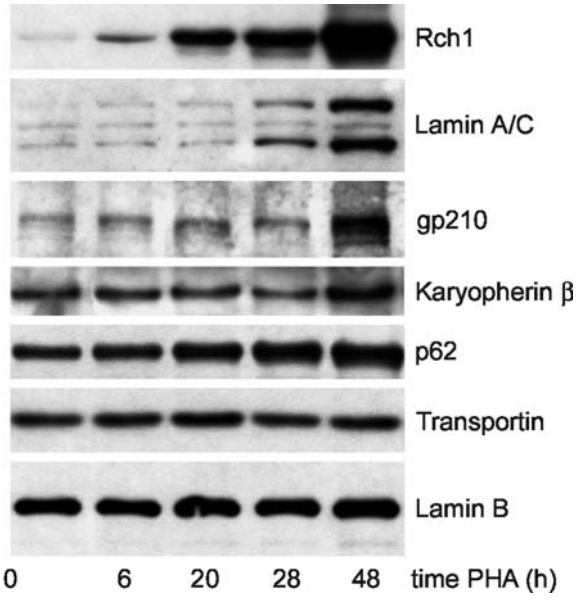


Fig. 1 Differential expression of nuclear envelope and nuclear transport component proteins during human lymphocyte activation for 48 h with phytohemagglutinin (*PHA*). Equal numbers of cells from each time treatment were loaded into each lane, separated by SDS-polyacrylamide gel electrophoresis and probed by immunoblotting with antibodies against the nuclear transport factors importin α (*Rch1*), importin β (*Karyopherin* β) and transportin, the nuclear lamina components lamin B and lamins A and C (*Lamin A/C*), and the nuclear pore proteins p62 and gp210. The level of expression of the different components ranged from the dramatic increase in Rch1 or lamins A and C, to a moderate enhancement of gp210, importin β and p62 and to the nearly constant levels of lamin B and transportin after 48 h of PHA treatment

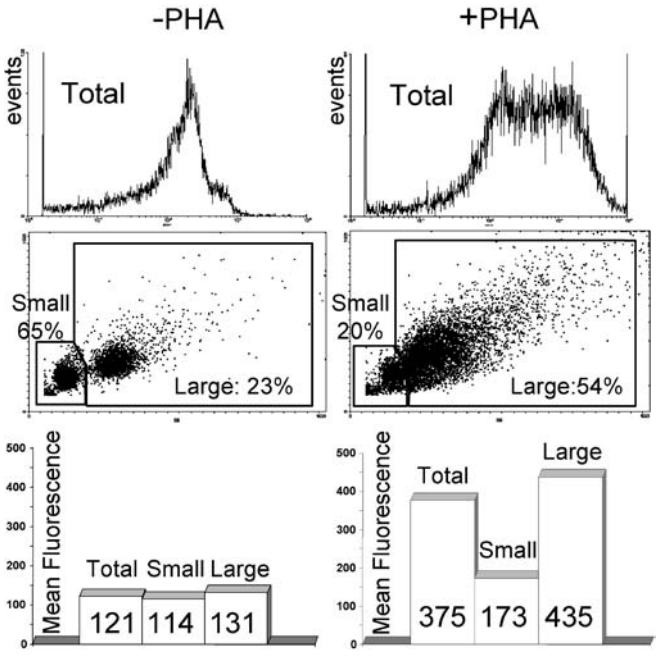
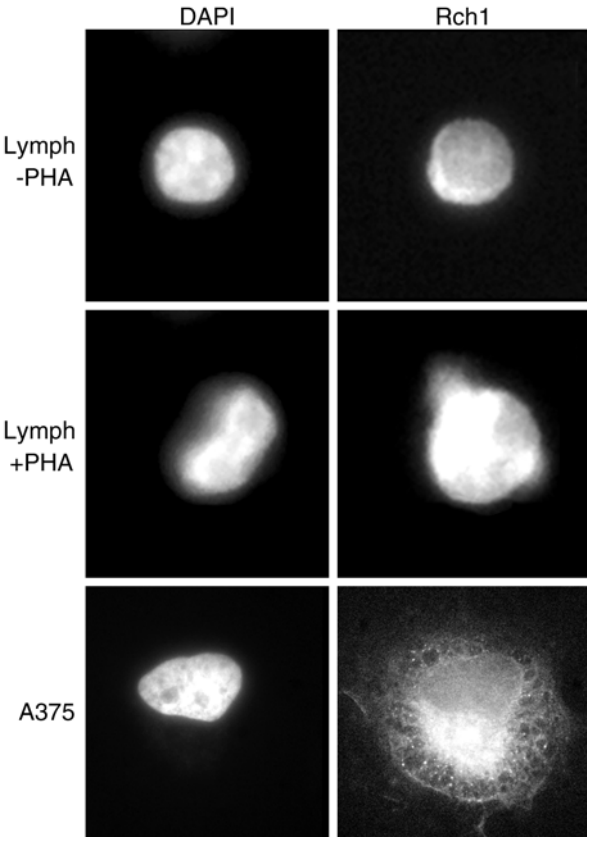


Fig. 2 Flow cytometry analysis of importin α (Rch1) levels in human peripheral blood cells treated (+*PHA*) or untreated (−*PHA*) with PHA for 48 h. Fluorescence (*top histogram*) was recorded in arbitrary units on a logarithmic scale for total cells or for two populations (large and small cells) obtained from the forward scatter versus side scatter biparametric analysis of the total population (*middle histogram*). PHA treatment leads to a large increase in the levels of Rch1 (mean fluorescence 121 vs 375, on a logarithmic scale). This enhancement is mainly due to the increased expression of Rch1 in large cells (131 vs 435, *bottom graph*)



By conventional immunofluorescence microscopy we observed the localization of Rch1 to be mainly cytoplasmic in both resting and active cells (Fig. 3). In accordance with the flow cytometry data, PHA-activated lymphocytes showed a significant increase in fluorescence intensity. In parallel studies, we observed that in A375, a human melanoma cell line, Rch1 stained brightly the juxtanuclear region, but it was also detected in strands throughout the cytoplasm and in the plasma membrane (Fig. 3). In order to determine the precise subcellular distribution of the nuclear transport components, we performed immunoelectron microscopy studies. All lamins and the nuclear pore proteins p62 and gp210 were detected at their expected location in the nuclear envelope, and no significant differences in the localization of either of these proteins in relation to the cell metabolic state could be observed. The soluble factors importin β and transportin, which are known to interact directly with the

Fig. 3 Immunofluorescence detection of importin α Rch1 in human lymphocytes (*Lymph*) and in A375 cells. The location of Rch1 in human lymphocytes is mainly cytoplasmic. *Left panels* Cell nuclei localized by DNA staining with 4′,6-diamidino-2-phenylindole (*DAPI*). A significantly higher accumulation of importin α Rch1 in a restricted area of the cytoplasm can be detected after 48 h PHA treatment (*right panels*). In the human melanoma cell line A375, Rch1 localizes to the juxtanuclear region, in cytoplasmic dots and in a strand-like pattern reaching the plasma membrane

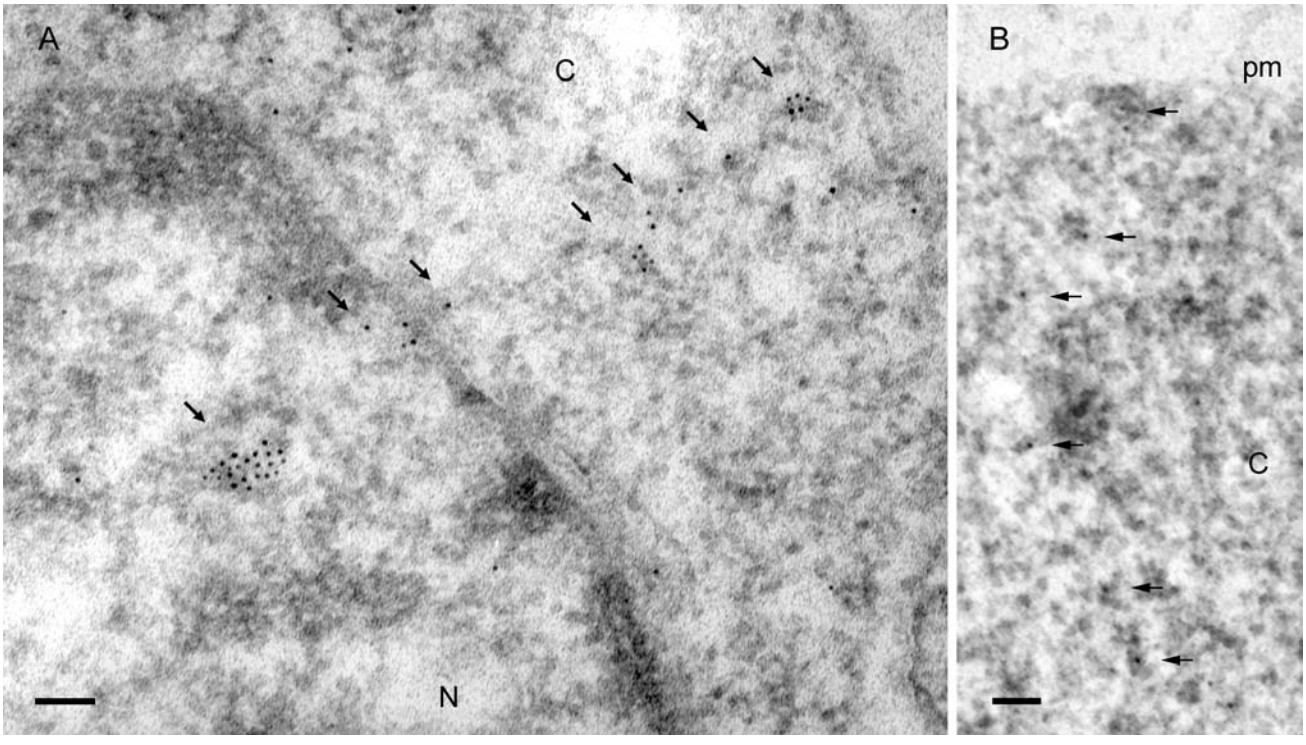


Fig. 4A, B Localization of importin α Rch1 in cytoplasmic tracks in human lymphocytes and A375 cells. **A** Post-embedding immunoelectron detection of Rch1 in human lymphocytes was performed as described in Materials and methods. Tracks of gold particles detecting Rch1 could be observed in a linear distribution from the cytoplasm (*C*), across the nuclear membrane into the nuclear interior (*N*). **B** Cytoplasmic tracks (*arrows*) reaching the plasma membrane (*pm*) could also be observed in A375 cells. *Bars* represent 100 nm

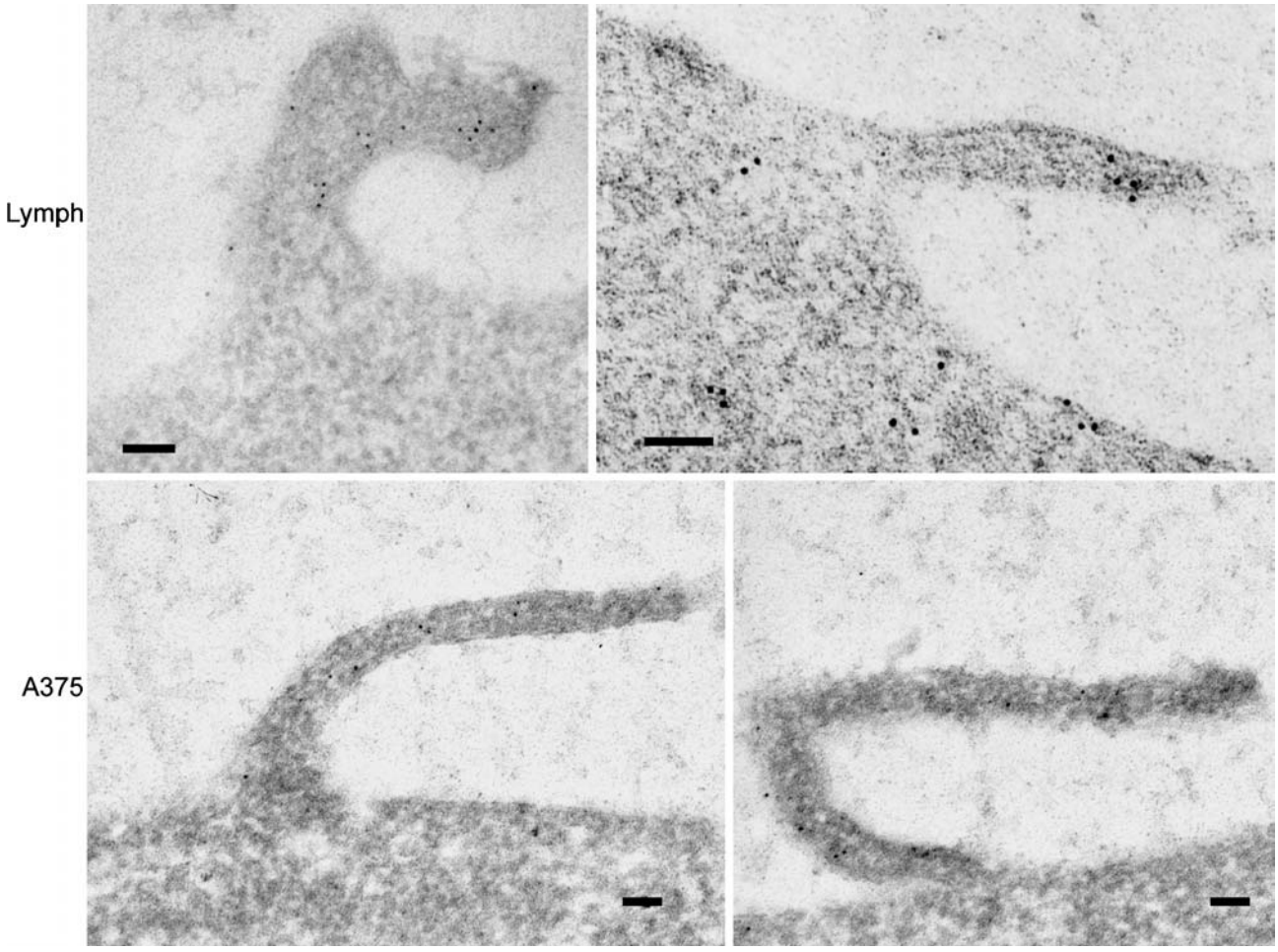


Fig. 5 Importin α Rch1 is present along cytoplasmic projections and microvilli. Gallery of electron micrographs of human lymphocytes (*Lymph*) and melanoma cells (*A375*) immunolabeled with anti-Rch1 antibodies. A detailed analysis of microvilli at the cellular surface shows the presence of importin α Rch1 in a linear distribution up to the tip of the cytoplasmic projections. *Bars* represent 100 nm

nuclear envelope transport machinery, were localized mainly in the nuclear pores and the nuclear interior (data not shown). In contrast, detection of Rch1 by electron microscopy revealed that this NLS receptor was mainly localized in the cytoplasm of both active and resting lymphocytes, in agreement with our observations using immunofluorescence. Additionally, Rch1 could be observed in linear tracks from the cytoplasm into the nucleus both in lymphocytes and A375 cells (Fig. 4). Surprisingly, in microvilli and in cytoplasmic projections, Rch1 labeling was clearly evident in a linear distribution along the axis of the microvilli (Fig. 5). Furthermore, we also observed Rch1 to be strikingly present at the cellular surface, in close contact with the plasma membrane (Fig. 6).

To analyze further the intracellular distribution of Rch1, we quantified the number of gold particles in resting and active lymphocytes (Table 1). Scrutiny of the gold particles showed that, in inactive cells, 84% of the total Rch1 labeling was cytoplasmic, of which 17% was associated with the plasma membrane. Only 8% of the labeling was nucleoplasmic in inactive cells, while another 8% was associated with the nuclear envelope (including both cytoplasmic and nuclear sides of the nuclear membrane). After lymphocyte activation, we observed a remarkable change in the subcellular distribution of Rch1 (Table 1). There was a highly significant

Table 1 Subcellular localization of Rch1 in resting (–PHA) and 48 h phytohemagglutinin-activated (+PHA) lymphocytes by immunoelectron microscopy. Quantification of gold particles was performed as described in Materials and methods. Results are given as a percentage of total number of gold particles (*n*)

	% Rch1 localization	
	–PHA	+PHA
Cytoplasm	84	75
Plasma membrane	*17*	*34*
Cytosol	*67*	*41*
Nuclear envelope	8	9
Nucleoplasm	8	16
	100% (*n* =987)	100% (*n* =849)

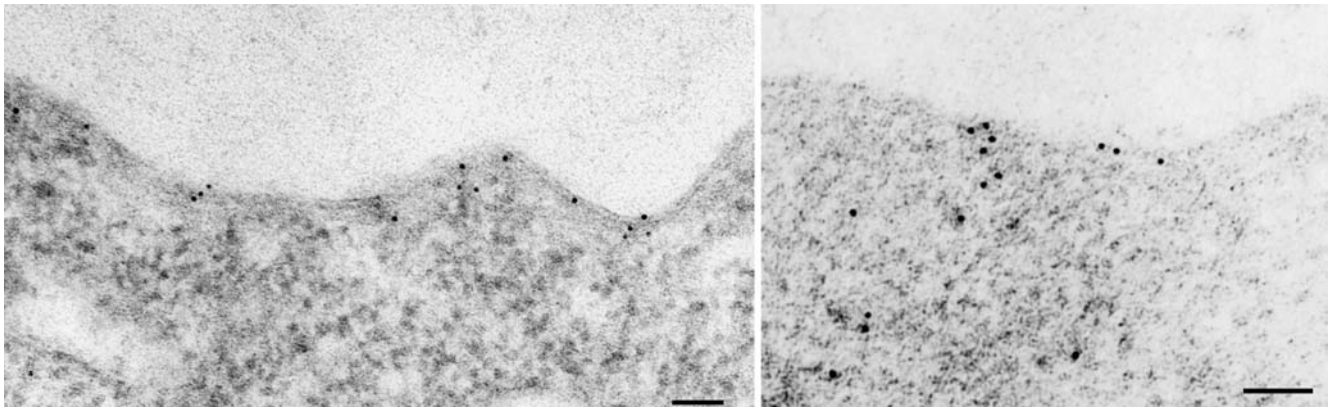


Fig. 6 Immunoelectron microscopy localization of importin α Rch1 at the plasma membrane. Ultrathin sections of Lowicryl-embedded peripheral blood lymphocytes were incubated with antibodies against importin α Rch1. A significant proportion of this nuclear localization sequence receptor was located in close association with the plasma membrane. *Bars* represent 100 nm

(P<0.001) redistribution of the cytoplasmic labeling, which decreased from 84% to 75%. Interestingly, this change was mainly due to the doubling of Rch1 associated with the plasma membrane (17% vs 34%) and nucleoplasm (8% vs 16%). Surprisingly, the levels of Rch1 at the nuclear envelope remained nearly constant (8% vs 9%) during lymphocyte activation. Thus, during lymphocyte activation, Rch1 appears to redistribute from its steady state localization in the cytoplasm to the plasma membrane and to the nuclear interior.

Discussion

In this study we observed that importin α Rch1 localizes at the plasma membrane and that its level of expression increases dramatically in response to the activation of human lymphocytes with PHA.

To accomplish the task of activation, the lymphocyte nucleus must undergo profound morphological and metabolic modifications, which require structural and regulatory components to be transported across the nuclear envelope. We observed that the levels of the nucleoporins p62 and gp210 as well as the import factor importin β seem to increase at a similar ratio, suggesting an overall activation of the import machinery.

We cannot rule out the possibility that the enhanced expression of Rch1 may simply lead to a general increase in the import of the newly synthesized proteins required for the structural nuclear changes occurring during PHA activation, since Rch1 binds to most NLSs (Miyamoto et al. 1997), while other proteins already present in the cytoplasm could be transported by more specific importin α isoforms.

However, in spite of nuclear enlargement, the levels of lamin B remained unchanged during the 48 h PHA treatment, while levels of lamins A and C increased only after 20 h of activation. Surprisingly, the levels of transportin, an importin β-related factor that participates in hnRNP transport (Pollard et al. 1996), remained constant during the process of activation. This result suggests that the transport rate of proteins dependent on transportin may be unaltered during PHA stimulation. On the other hand, the low levels of expression of Rch1 in resting lymphocytes indicate that Rch1 may not participate in basal transport processes in resting lymphocytes. Similar results concerning Rch1 expression were reported by Nadler et al. (1997), who found that the importin α homologs hSRP1 and hSPRP1α (Rch1) were upregulated in normal lymphocytes following metabolic activation.

Regarding the molecular mechanisms of nuclear transport, the enhanced expression of Rch1 after 48 h activation implies an imbalance in the levels of the α (Rch1) and β (karyopherin) importins, which are thought to interact in a 1:1 stoichiometric ratio (Hübner et al. 1997; Görlich and Kutay 1999; Catimel et al. 2001). There are several plausible explanations for this observation. (1) importin β is involved in many processes that do not involve importin α (nuclear import of proteins that bind directly to importin β; Johnson-Saliba et al. 2000; Schedlich et al. 2000), which would decrease the availability of importin β for importin α. (2) Importin α could be imported into the nucleus in an importin β-independent manner, as has been recently described (Miyamoto et al. 2002). (3) The activity or the localization of importin α could also be regulated by post-translational modifications (Bannister et al. 2000), adding another step to the regulation of nuclear transport. (4) The subcellular redistribution of Rch1 between resting and activated lymphocytes should involve specific changes in the regeneration of each factor to participate in new cycles of transport. In this context, the increased localization of Rch1 at the plasma membrane after activation suggests that the functions of Rch1 could be regulated depending on its subcellular distribution. This hypothesis is reinforced by the observation that the aggregation state of importin α depends on the presence or absence of NLSs (Fischer et al. 1997; Smith and Raihkel 1998; Percipalle et al. 1999). The subcellular localization of Rch1 at microvilli and in cytoplasmic tracks is also

reminiscent of the recently reported association of importin α with the cytoskeleton in tobacco protoplasts (Smith and Raihkel 1998). Unfortunately, little is known about the interaction of cargoes with the nucleocytoplasmic transport machinery. One possibility is that importin α could be involved in the recognition of karyophilic proteins that remain anchored as latent factors complexed with the cytoskeleton in specific regions of the cytoplasm. The upregulation of a particular importin α isoform in response to extracellular signals associated with lymphocyte activation could activate the nuclear import of specific karyophilic proteins.

We observed that the nuclear content of Rch1 doubled after 48 h lymphocyte activation. Similarly, the α2 pendulin in *Drosophila* embryos was found predominantly in the cytoplasm during interphase (Küssel and Frasch 1995), whereas this importin accumulates in the nucleus prior to mitosis (Küssel and Frasch 1995; Török et al. 1995). Curiously, the importin α3 isoform has been reported to have a nuclear localization during interphase (Máthé et al. 2000), in contrast to that of α2, indicating that these proteins may operate in independent processes. It has also been found that each importin α isoform is expressed during a specific and limited period of spermatogenesis (Giarré et al. 2002). Interestingly, mutations in this importin α isoform led to over-proliferation of hematopoietic cells in *Drosophila* larvae (Küssel and Frasch 1995) and abnormal proliferation in a breast cancer cell line (Kim et al. 2000), raising the possibility that importin α may act as a cell tumor suppressor. These results are also supported by the enhanced expression of importin α detected in several human leukemia cell lines in a manner proportional to their degree of differentiation (Nadler et al. 1997), as well as by the appearance of lamins A and C during lymphocyte activation (see Fig. 1), which is generally associated with processes of differentiation and proliferation (Moir et al. 2000; Venables et al. 2001). Recent studies by Köhler et al. (2002) have also revealed differential expression of importin α isoforms after the induction of differentiation and proliferation of several human tumor cell lines. Thus, the control of the expression, activity and localization of nuclear import factors could represent an important level of gene regulation integral to cellular processes such as differentiation and transformation.

One of the most exciting research targets in the near future will be to elucidate the mechanisms linking different signal transduction pathways with nucleocytoplasmic transport. Although the final destination of many signal transduction pathways is the nuclear interior to modulate gene expression, little is known about the participation of nuclear transport in these processes. Recent studies have reported that, in response to an extracellular stimulus, transmembrane receptors from the plasma membrane are internalized into the nucleus where they modulate the expression of specific genes (Lin et al. 2001; Reilly and Maher 2001). Moreover, karyopherin α2 has been detected at the plasma membrane in hepatic cells, where it interacts specifically with the glucose transporter GLUT2 (Guillemain et al. 2002). In this context, the subcellular localization of Rch1 at the plasma membrane raises the possibility of a novel role for Rch1, not only in nuclear import across the nuclear envelope, but also in providing a direct link in the signal transduction of extracellular stimuli from the plasma membrane to the nucleus.

Overall, these results suggest that importin α isoforms might not function exclusively as passive NLS receptors, but may also participate actively in signal transduction and nuclear transport events during embryonic development, cellular differentiation and cell cycle control.

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