Ligands bound to specific receptors on the cell surface are internalized by receptor-mediated endocytosis. Once they have reached endosomes, receptors can either recycle back to the plasma membrane or be delivered to other intracellular compartments.

Different groups have assessed the possible role of the cytoskeleton in endocytosis. The actin cytoskeleton, long thought not to be involved in endocytosis, has recently been shown to play a role at different steps along the endocytic pathway, in polarized and non-polarized cells and in yeast (Gottlieb et al., 1993; Durrbach et al., 1996a,b; Geli and Riezman, 1996). Microtubules are involved in late steps of endocytosis, in the traffic between early and late compartments. When microtubules are disrupted, translocation of endosomes and lysosomes is inhibited (Matteoni and Kreis, 1987; Gruenberg et al., 1989). On the other hand, the role of microtubules in the early steps of receptor-mediated endocytosis remains controversial. Although most authors agree that microtubules do not participate in the first steps of entry or recycling (Kelly, 1990), inhibition of transferrin internalization by microtubule-disrupting drugs has also been reported (Jin and Snider, 1993; Thatte et al., 1994). Moreover, these studies were carried out on receptors which are internalized via the best described clathrin-coated pit pathway. Other pathways of entry exist (Hansen et al., 1991; reviewed by Lamaze and Schmid, 1995) and their sensitivity to microtubule depolymerization has not been documented.

Receptors entering by the clathrin-coated pit pathway carry internalization signals in their cytosolic tail allowing their recruitment in coated pits (Robinson et al., 1996). These signals are short peptides which have been classified in two groups, a tyrosine based motif and a di-leucine based motif (reviewed by Sandoval and Bakke, 1994). Receptors for the iron transporter, transferrin, are abundantly expressed at the cell surface of most cell types, from where they are internalized via clathrin-coated pits and vesicles. Their endocytosis is constitutive and very efficient, which makes them a suitable and widely used marker to study clathrin-mediated endocytosis. Their intracellular domain contains an endocytic signal, the amino acid sequence YTRF (Collawn et al., 1990; Jing et al., 1990), which is recognized by the p2 subunit of clathrin-associated adaptor complex AP-2 (Ohno et al., 1996), and leads to receptor recruitment into clathrin-coated pits. After internalization (t1/2 ~3 minutes), transferrin releases its iron in early endosomal compartments and recycles back with its receptor to the cell surface (t1/2 ~10 minutes), to be reused as an iron transporter (Ciechanover et al., 1983; Dautry-Varsat et al., 1983; Klausner et al., 1983).

The cytokine interleukin 2 (IL2) is produced by activated helper T lymphocytes and stimulates proliferation and effector functions of a variety of cells of the immune system (Minami et al., 1993). High-affinity IL2 receptors (Kd =10-100 pM) consist of three distinct subunits, the α, β and γ chains, that are associated in a noncovalent manner (Minami et al., 1993). One of the early events following IL2 binding to high-affinity receptors on the cell surface is the internalization of IL2 entry was unaffected by microtubule disruption, as previously reported. Unlike the case for transferrin, internalization of IL2 receptors was not affected by depolymerization of the microtubule cytoskeleton in hemopoietic cell lines. These results show that IL2 and transferrin receptors do not have the same endocytic properties and support our previous conclusion that these receptors follow different pathways of endocytosis.

Key words: Transferrin, Receptor, Lymphocyte, Coated-pit independent endocytosis, Cytoskeleton

SUMMARY

Microtubule depolymerization inhibits clathrin coated-pit internalization in non-adherent cell lines while interleukin 2 endocytosis is not affected

Agathe Subtil and Alice Dautry-Varsat*

Unité de Biologie des Interactions Cellulaires, URA CNRS 1960, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France

*Author for correspondence (e-mail: adautry@pasteur.fr)
receptor complexes (Fujii et al., 1986; Weissman et al., 1986; Duprez et al., 1988). We have shown that these receptors may be internalized by a clathrin-independent pathway, as they are still internalized when clathrin-coated pit endocytosis is inhibited (Subtil et al., 1994). After endocytosis, the components of this multimolecular receptor have different intracellular fates: one of the chains, α, recycles to the plasma membrane, while the others, β and γ, are routed to late endocytic compartments (Hémar et al., 1995; Subtil et al., 1997).

Activated T lymphocytes and natural killer (NK) cells which express IL2 receptors are circulating cells and grow in suspension in vitro. It has been shown that in leukocytes the microtubule cytoskeleton has different properties from the cytoskeleton of adherent cells and is more dynamic (Ding et al., 1995). Here, we show that depolymerization of the microtubule cytoskeleton inhibits internalization of transferrin receptors in several hemopoietic cell lines grown in suspension, as opposed to what is observed in adherent cells. The entry of IL2 receptors was not affected by this treatment. This result supports our finding that this receptor may use a clathrin-independent internalization pathway.

MATERIALS AND METHODS

Cells

IARC 301.5 is a subclone from a cell line derived from a human T lymphoma, which expresses high- and low-affinity IL2 receptors (Duprez et al., 1985, 1988). YT12881 and YT2C2, subclones from the NK cell line YT, were obtained from Dr Kendall Smith (Dartmouth Medical School, NH, USA; Teshigawara et al., 1987). YT12881 was activated by incubating the cells with 0.1 mg/ml interleukin 1 (IL1; ImmunogeneXx, CA, USA) for 20 hours at 37°C. After activation, they express about 12,000 high-affinity IL2 receptors. K562 is a human erythroleukemia cell line. These cells were grown in suspension in RPMI 1640, 10% decomplemented fetal calf serum, 10 mM Heps, pH 7.2, supplemented with 2 mM L-glutamine, and with 1.5 mg/ml G418 for stably transfected K562 cells. Three non-hemopoietic cell lines were also tested: BHK21, a baby hamster kidney cell line; HepG2, a human hepatoma cell line; and HeLa, a human cervix carcinoma cell line. These cells were grown in DMEM, 10% decomplemented fetal calf serum, supplemented with 2 mM L-glutamine.

Monoclonal antibodies and reagents

Monoclonal antibody 7G7B6, directed against the α chain of IL2 receptors, was obtained from the American Tissue Culture Collection (Rockville, MD). Monoclonal antibody 561, directed against the β chain of IL2 receptors, was a kind gift from Dr R. Robb (Dupont Merck Pharmaceutical Co., Wilmington, DE; Voss et al., 1993). Human transferrin (Sigma) was loaded with iron as previously described (Ciechanover et al., 1983). Purified recombinant IL2 (Sanofi, France), transferrin and antibodies were radiolabeled with 125I choline by the chloramine T method to specific activities of 10 μCi/μg, 0.2 μCi/μg and 15 μCi/μg, respectively, as previously described (Subtil et al., 1994). Nocodazole and colchicine (Sigma) were aliquoted at 10 mM in dimethylsulfoxide (DMSO) and used at 10 μM; in all control experiments, 0.1% DMSO was added to the cells.

Plasmids and cell transfection

The plasmid pKCRβ, coding for the IL2 receptor β chain, kindly provided by Dr T. Kono (Osaka University, Japan), was subcloned in NT, a kind gift from Dr C. Bonnerot (Institut Curie, Paris, France), and the construct was named β27. The construct β27 was generated by PCR by inserting a stop codon after the 27th amino acid of the cytosolic part of the protein (assuming that asparagine is the first cytosolic amino acid) (Subtil et al., 1997). β27F24Y was constructed from β27 by substitution of phenylalanine 24 to tyrosine.

The plasmid T-XO, a kind gift from Dr P. Cosson (Geneva, Switzerland), is a modified version of the IL2Rα cDNA in the pCDM8 vector (Subtil et al., 1997). This plasmid was further modified by inserting a sequence coding for the transferrin receptor YTFR internalization signal, in the cytosolic part of α, and was subcloned in the NotI/SacI cloning sites of NT and named α27 (Subtil et al., 1997).

RESULTS

Disruption of the microtubule cytoskeleton inhibits transferrin endocytosis in cell lines grown in suspension

Several microtubule-disrupting agents, such as nocodazole, can be used to depolymerize the microtubule cytoskeleton. Transferrin internalization was measured after a 30 minute incubation in 10 μM nocodazole. Transferrin receptor recycling takes place very rapidly after internalization (Ciechanover et al., 1983; Dautry-Varsat et al., 1983). To measure the actual entry of transferrin, without recycling, one must use a very short time of incubation with the ligand (≤3 minutes). Transferrin internalization was measured without preincubating the cells at 4°C since it has been shown that exposure to the cold disrupts the microtubule cytoskeleton (Cassimeris et al., 1986) and would thus affect the control. Transferrin internalization was inhibited by about 40% in nocodazole treated YT cells (Fig. 1). The kinetics show a short lag before internalization (Fig. 1), likely due to the time necessary for transferrin binding. The effect of microtubule depolymerization was similar to that on transferrin entry reported in another cell line grown in suspension, K562 (Jin and Snider, 1993; Thatte et al., 1994). Altogether, these results are surprising, as microtubules are usually considered not to be involved in the early steps of endocytosis. As most studies have been carried out on adherent cell lines, we tested the effect of microtubule-disrupting drugs on different cell lines, adherent or grown in suspension. Cells were treated with 10 μM nocodazole for 30 minutes before measuring 125I-transferrin uptake for three minutes (Fig. 2). Under these conditions, we verified by immunofluorescence microscopy, using anti-tubulin antibodies, that the microtubule cytoskeleton was disrupted in all cell lines (not shown). In agreement with
Microtubule-disruption and endocytosis

Microtubule-disruption and endocytosis

The entry of αY, a chimera bearing the transferrin receptor endocytic motif, is also impaired by microtubule disruption

To see if the impairment of transferrin entry in the cells grown in suspension tested was due to a general defect in clathrin-mediated endocytosis, we designed a new marker of this pathway, αY. The α chain of the interleukin 2 (IL2) receptor has a short cytosolic tail of 13 amino acids, is not internalized by itself (Fujii et al., 1986; Weissman et al., 1986) and has been often used to prepare chimeric receptors (Letourneur and Klausner, 1992; Marks et al., 1995; Dittrich et al., 1996). We have constructed a chimera, αY, in which a short amino acid sequence of the transferrin receptor, including the YTRF internalization signal, was added to the cytosolic sequence of α. The construct was stably transfected in K562 cells, and we have previously shown that it is internalized very efficiently, as determined using iodinated anti-α antibody 7G7B6 (Subtil et al., 1997). After disruption of the microtubule network by nocodazole treatment, the endocytosis of αY probed with this antibody was inhibited by about 40% (Fig. 3). This inhibition was similar to that observed for transferrin entry. Therefore, disruption of the microtubule cytoskeleton inhibits entry of a receptor designed to be internalized via coated-pits.

The entry of IL2 is not affected by microtubule depolymerizing treatment

We had shown in an earlier study that interleukin 2 receptors may be endocytosed by a clathrin-independent pathway, as they are still internalized when clathrin-coated pit endocytosis is inhibited (Subtil et al., 1994).
We studied the effect of microtubule disruption on IL2 endocytosis in two cell lines expressing IL2 receptors. IARC 301.5 cells constitutively express about 3,000 high-affinity IL2 receptors and YT 12880 cells express about 12,000 high-affinity receptors after activation by IL1. The cells were incubated for 30 minutes in 10 μM nocodazole at 37°C before measuring 125I-IL2 or 125I-transferrin endocytosis for 10 or 2.5 minutes, respectively. These incubation times were chosen so that the kinetics of entry for each ligand should be within the linear range: at 37°C, the rate of endocytosis of IL2 is about three times slower than that of transferrin (see Fig. 5). Nocodazole treatment did not affect IL2 internalization in these cells, while in parallel experiments, transferrin entry was inhibited by about 40% (Fig. 4). The same result was obtained when 10 μM colchicine was used instead of nocodazole, when 125I-transferrin entry was measured in the presence of unlabeled IL2, or when 125I-IL2 was measured in the presence of unlabeled transferrin (not shown).

Another interesting difference between IL2 and transferrin endocytosis was found when cells were pretreated at 4°C before measuring endocytosis at 37°C. We measured the rate of 125I-transferrin and 125I-IL2 entry in IARC 301.5 cells, at 37°C, after a 90 minute preincubation at 4°C. The 125I-transferrin entry rate was 45% of that in control cells (not exposed to the cold before measuring endocytosis), while 125I-IL2 entry was only slightly reduced, by about 15% (Fig. 5). It is known that exposure of cells to cold temperatures results in microtubule depolymerization (Cassimeris et al., 1986). Indeed, we have observed, by immunofluorescence microscopy using anti-tubulin antibody, that after a 90 minute incubation at 4°C, the microtubule cytoskeleton was depolymerized in IARC 301.5 cells (not shown). The observed inhibition of transferrin internalization may therefore be due to microtubule disruption, although the effect of exposing cells to the cold is not restricted to microtubules.

Taken together, these experiments show that endocytosis of IL2 and transferrin are affected differently by microtubule disrupting drugs and by exposure of cells to the cold.

**Addition of a clathrin-coated pit like internalization motif to the β chain of the IL2 receptor confers sensitivity to nocodazole treatment**

We had previously shown that the IL2 receptor β chain as well as a truncated form of β, β27, containing only the first 27 cytosolic amino acids starting from the membrane, can be internalized by themselves, in the absence of α, γ or IL2 (Hémar et al., 1994; Subtil et al., 1997). We constructed a modified form of β27, β27F24Y, in which phenylalanine 24 was mutated to a tyrosine (Fig. 6a). In this construct, the last four
cytosolic amino acids are YSQL. This motif fulfills the requirements for the recognition of tyrosine-based endocytic signals by AP-2 complexes, as it has a tyrosine-polar-polar-hydrophobic amino acid sequence (Boll et al., 1996). In stably transfected K562 cells, endocytosis of the β27F24Y construct was faster than that of β27 (Fig. 6b), suggesting that β27F24Y is indeed efficiently internalized by clathrin-coated pits. Incubation of the cells for 30 minutes in 10 μM nocodazole did not affect the internalization of βwt or β27, while β27F24Y entry was inhibited by 30% (Fig. 6c). Thus, the addition of a clathrin-coated pit like endocytic signal was sufficient to confer nocodazole sensitivity to the internalization of β27.

**DISCUSSION**

We have compared the internalization of IL2 and transferrin in cell lines in which the microtubule cytoskeleton had been disrupted by different methods. In cells expressing the IL2 receptor, derived from the hemopoietic lineage and growing in suspension, microtubule disruption had no effect on IL2 endocytosis. However, transferrin entry was inhibited by about 40%. We also studied the internalization of αγ, the α chain of the IL2 receptor to which the YTRF endocytosis signal of the transferrin receptor was added in the cytosolic tail. After microtubule disruption, its internalization was inhibited to the same extent as that of transferrin.

The role of the microtubule cytoskeleton in endocytosis has been studied by several authors. It is clear that late steps of endocytosis depend on an intact microtubule network (Matteoni and Kreis, 1987; Gruenberg et al., 1989). Several independent studies have also shown that the first steps of clathrin-dependent endocytosis are not affected by microtubule disrupting drugs in different adherent cell lines (Oka and Weigel, 1983; Wolkoff et al., 1984; Caron et al., 1985; Gruenberg et al., 1989; Hunziker et al., 1990). This is also what we observed in this study with HeLa, HepG2 and BHK21 cell lines. However, in three lines, of lymphocytic, NK and erythroid origin, with a spherical morphology, transferrin internalization was inhibited. This has also been observed by two groups studying transferrin internalization in K562 cells (Jin and Snider, 1993; Thatte et al., 1994). Our finding that the entry of αγ is also impaired by microtubule disruption indicates that this treatment more generally affects endocytosis via clathrin-coated pits. It is worth noting that this study has been performed at early times of endocytosis; it is thus very likely...
that the decrease in internal transferrin is due to effects of cytoskeleton disruption on the initial steps of endocytosis and not on recycling.

The reason why microtubule disruption affects endocytosis in some cell lines of hemopoietic origin and grown in suspension is not clear. The discrepancy between such cells and adherent cell lines may be explained by differences in the cytoskeletal organization in these different cell types. For instance, the cortical cytoskeleton is more stably organized in adhering cells because of their focal adhesions. Also, there are numerous post-translational modifications of microtubules, which differ depending on the cell type and affect their interactions with other cellular components and their resistance to microtubule-disrupting drugs (Kreis, 1987). Microtubule-associated proteins are also somewhat cell-specific. Strikingly, microtubules are more dynamic in hemopoietic cells, in which their half-life is only about 30 seconds as compared to 15-20 minutes in other cells (Ding et al., 1995). Because of this instability, microtubule-depolymerizing treatments are probably much more efficient in hemopoietic cells, which may account for the differences observed between cell types.

Microtubules underlie the actin cortical cytoskeleton and do not form a very dense network. It is unlikely that they may participate directly in the formation of clathrin-coated vesicles. However, they may affect membrane plasticity, especially in cells such as the ones studied here which are malleable. In these cells, modification of the cell plasticity could affect two early steps in clathrin-coated pit endocytosis: access of receptors to the pits and vesicle formation.

At steady state, 90% to 94% of transferrin receptors on the plasma membrane are outside of coated pits in the seven cell lines which have been examined (Miller et al., 1991; Hansen et al., 1992). To reach coated pits, transferrin receptors must be mobile on the plasma membrane. It has already been shown that receptor mobility in the plasma membrane can be a limiting parameter for endocytosis (Gonçalves et al., 1993; Paccaud et al., 1993). Recent single-particle tracking experiments have shown that the plasma membrane is compartmentalized in small domains with regards to transferrin and asialoglycoprotein receptor movement and that this membrane organization is disturbed when microtubules are disrupted (Sako and Kusumi, 1994, 1995).

Interestingly, IL2 receptor endocytosis was not affected by microtubule disruption using drugs, nocodazole or colchicine, or low temperature incubation. This was shown in two lymphocytic lines expressing high-affinity IL2 receptors and in K562 cells stably transfected with the IL2 receptor β chain or a truncated form of β, β27. We have previously reported that IL2 endocytosis proceeds when the clathrin-dependent pathway is inhibited (Subtil et al., 1994). The different sensitivity of IL2 and transferrin entry to microtubule disruption may reflect differences in their internalization pathways. We addressed this issue by creating a signal predicted to be favorable for clathrin-dependent internalization, YSQL, in the β27 chain. Indeed, tyrosine-polar-polar-hydrophobic sequences interact with AP-2, with leucine being the most favorable residue at position Y + 3 (Boll et al., 1996). This chimera was internalized faster than β27 suggesting that it was indeed internalized by clathrin-coated pits. Interestingly, its rate of entry was decreased by microtubule disruption, unlike the case for β27.

Taken together, our results indicate that clathrin-dependent and independent endocytosis are differently affected by microtubule disruption. One possibility is that formation of clathrin-coated vesicles is inhibited by microtubule depolymerization, while clathrin-independent vesicle formation is not. Another way to study clathrin-dependent and independent internalization is to follow fluid phase endocytosis (Cupers et al., 1994; Damke et al., 1994). In K562 cells, transferrin internalization was inhibited by nocodazole treatment, but fluid phase endocytosis of horseradish peroxidase was not (Jin and Snider, 1993). This is in agreement with our results and is in favor of the existence of several endocytosis pathways displaying different dependence on the microtubule network.

The other possibility raised by our results, as discussed above, is that access to the coated-pits is limiting for transferrin receptors when microtubules are disrupted because they have to cross membrane barriers to reach coated pits (Sako and Kusumi, 1994). If access to areas of the membrane competent for endocytosis is not limiting for IL2 receptors, it could mean that membrane areas involved in clathrin-independent endocytosis are more widely distributed on the membrane. It is worth noting that the cytosolic tail of the IL2 receptor β chain interacts with a number of polypeptides including the γ chain and several intracellular kinases (Minami et al., 1993). Thus, if microtubule polymerization simply affected the mobility of all membrane proteins by trapping their cytosolic tail, one would expect the IL2 receptor to be less mobile than the transferrin receptor, which would lead to a result opposite to what we observe.

Clathrin-independent endocytosis is still poorly understood. Whether there is one or several clathrin-independent pathways is unknown. IL2 endocytosis (this study) and fluid phase endocytosis (Jin and Snider, 1993) are not affected by microtubule disruption in K562 and lymphocytic cells, while clathrin-coated pit endocytosis appears to be inhibited. It would be interesting to know if the endocytosis of other molecules internalized in a clathrin-independent manner is also resistant to microtubule depolymerization in these cells. If not, this would indicate that there may be more than one clathrin-independent endocytosis pathway. In any case, this study further supports the view that IL2 receptors do not display the same endocytic characteristics as receptors internalized via clathrin-coated pits.

We are grateful to Drs P. Cosson and T. Kono for providing plasmids T-XO and pdKCRβ, respectively; to Eric Sénéchal for excellent technical help; and to Drs A. Benmerah, Ch. Lamaze and D. Ojcius for critical reading of the manuscript. This work was supported by the CNRS and by Human Frontier Science Program.

REFERENCES


