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# Rapid endocytosis of interleukin 2 receptors when clathrin-coated pit endocytosis is inhibited

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## SUMMARY

**The cytokine interleukin 2 (IL2) is produced by activated helper T lymphocytes and modulates the growth and activity of cells expressing high-affinity surface IL2 receptors that transduce its signaling. After ligand binding to receptors on the plasma membrane, receptor-ligand complexes are rapidly endocytosed and IL2 is degraded in acidic compartments. The best known receptor-mediated endocytosis pathway involves clathrin-coated pits. Receptors that carry an internalization signal recognized by adaptors on the cytosolic side of the plasma membrane are clustered into the coated pits and enter cells very efficiently. Many receptors use this pathway, but other endocytic pathways have also been reported, for ricin, EGF and insulin, for instance, which seem to be less efficient than the coated one. We compared the endocytosis of IL2 and its receptors to that of transferrin, a marker of the coated pit pathway. Under normal conditions, the kinetics of entry of IL2 was two times slower than that of transferrin. When internalization via coated pits was inhibited by two different methods, potassium depletion and cytosol acidification, endocytosis of IL2 and its receptors was only partly inhibited, while transferrin entry was strongly affected. Treatment with the cationic amphiphilic drug chlorpromazine, which induces a redistribution of a clathrin-coated pit component, AP-2, to endosomes, reduced transferrin, but not IL2 internalization. Thus, unexpectedly, this cytokine and its receptors can still be rapidly endocytosed in the absence of functional clathrin-coated structures. We propose a model for receptor-mediated endocytosis that may account for these results and published data on other receptors.**

Key words: endocytosis inhibition, endocytic pathway, transferrin

## INTRODUCTION

The interaction of interleukin 2 (IL2) with high-affinity IL2 receptors on the cell surface of T lymphocytes triggers intracellular events that lead to T cell proliferation (Smith, 1989; Waldmann, 1991; Takeshita et al., 1992; Minami et al., 1993; Taniguchi and Minami, 1993; Voss et al., 1993). One of the early events that follows the binding of IL2 to such receptors is the internalization and degradation of the ligand in acidic compartments (Robb et al., 1981; Duprez and Dautry-Varsat, 1986; Duprez et al., 1988, 1994; Smith, 1989; Ferrer et al., 1993). The high-affinity receptor complex ($K_d$ ~ 10-100 pM) consists of three distinct receptor components, the α chain (50-55 kDa), the β chain (70-75 kDa) and the γ chain (65 kDa), that are associated in a noncovalent manner (Waldmann, 1991; Taniguchi and Minami, 1993). Natural killer cells and activated T lymphocytes may also express intermediate ($K_d$ ~ 1 nM) or low-affinity ($K_d$ ~ 10 nM) IL2 receptors that do not contain α or β and γ, respectively (Smith, 1989; Waldmann, 1989). Both the β and γ chains, but not the α chain, belong to the cytokine receptor superfamily (Bazan, 1990; D'Andrea, 1994). Recently the γ chain was found to participate in the formation of high-affinity forms of IL4, IL7 and IL15 receptors (Kondo et al., 1993; Noguchi et al., 1993a; Russell et al., 1993; Giri et al., 1994). The β polypeptide is also a component of the IL15 receptor (Giri et al., 1994). The γ chain plays a critical role in thymic maturation of precursor human T lymphocytes, since patients suffering from X-linked severe combined immunodeficiency have a mutation in the gene encoding this chain (Noguchi et al., 1993b; DiSanto et al., 1994a,b). The molecular mechanism by which IL2 signaling is transduced is still unclear, and other chains associated with this receptor complex, such as the tyrosine kinases $p56^{lck}$, $p59^{fyn}$, $p53/56^{lyn}$ and JAKs may be implicated (Kim et al., 1993; Kobayashi et al., 1993; Taniguchi and Minami, 1993).

The best-characterized receptor-mediated endocytosis pathway involves, in its initial steps, the clustering of receptors and ligands in clathrin-coated pits on the plasma membrane, which invaginate and form clathrin-coated vesicles (Goldstein et al., 1985; Brodsky, 1988; Pearse and Robinson, 1990; Smythe and Warren, 1991). However, other receptor-mediated endocytosis pathways through non-clathrin-coated invaginations have also been described (reviewed by Watts and Marsh, 1992), such as for ricin (Moya et al., 1985; Sandvig et al., 1987), epidermal growth factor (EGF) and insulin in some cell lines (Lund et al., 1990; Backer et al., 1991), as well as for cholera and tetanus toxins (Montesano et al., 1982; Tran et al., 1987).

Endocytosis through clathrin-coated structures can be inhibited by potassium depletion (Larkin et al., 1983), cytosol acidification (Sandvig et al., 1987) and treatment with cationic amphiphilic drugs (Wang et al., 1993). As previously shown by electron microscopy, potassium depletion inhibits this pathway by blocking clathrin-coated pit formation (Larkin et al., 1986; Heuser and Anderson, 1989). Cytosol acidification prevents endocytosis via coated pits by 'paralyzing' clathrin lattices: they curve inward until they become almost spherical but remain attached to the plasma membrane (Sandvig et al., 1987; Heuser, 1989). After these treatments, adaptors are still aggregated at the plasma membrane (Hansen et al., 1993a) and adaptins are still associated with receptors, as shown in the case of the EGF receptor (Sorkin and Carpenter, 1993). More recently, it has been reported that cationic amphiphilic drugs cause a decrease in coated pits on the cell surface and clathrin lattices assembly on endosomal membranes. The authors propose that these drugs act by inhibiting the return to the plasma membrane, during the endocytic cycle, of a clathrin-coated pit component, AP-2 (Wang et al., 1993).

In this work we have used these methods to investigate the entry pathway of IL2 receptors in a T cell line and a natural killer cell line. The human T lymphocytic cells IARC 301.5 express about 3000 high-affinity IL2 receptors, internalize IL2 and proliferate in response to this growth factor (Duprez et al., 1985, 1988). The human natural killer cells YT 12881, activated by IL1, express about 12,000 high-affinity IL2 receptors and internalize IL2 (Teshigawara et al., 1987). We have compared the internalization of IL2 receptors to that of transferrin, a marker of the coated pit pathway, to that of ricin, which can enter cells by another pathway, and finally to fluid-phase endocytosis. We show that when endocytosis via clathrin-coated structures is impaired, IL2 receptors are still partly internalized as well as ricin and a fluid-phase marker, while tranferrin entry is inhibited. Therefore it appears that IL2 can enter cells when clathrin-coated endocytosis is inhibited. A model is presented that accounts for our results and published reports concerning endocytosis of other growth factor receptors, and previous data on endocytosis of membrane components.

## MATERIALS AND METHODS

### Cells and monoclonal antibodies

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 natural killer (NK) cell line YT, were obtained from Dr Kendall Smith (Dartmouth Medical School, NH, USA; Teshigawara et al., 1987). All cells were grown in RPMI 1640, 10% fetal calf serum, 10 mM HEPES, pH 7.2, supplemented with glutamine. YT12881 were activated by incubating the cells with 0.1 ng/ml IL1 (Immunogenex, CA, USA) for 20 hours at 37°C. After activation, they express about 12,000 high-affinity IL2 receptors.

Monoclonal antibodies 7G7B6, directed against the α chain of IL2 receptors (Rubin et al., 1985), and OKT9, directed against the transferrin receptor (Sutherland et al., 1981), were obtained from the American Tissue Culture Collection. The anti-IL2 receptor β chain monoclonal antibody 561 was a kind gift from Dr Richard Robb (Dupont Merck Pharmaceutical Co, DE, USA) (Voss et al., 1993).

### Potassium depletion of cells

Potassium depletion was carried out as described (Larkin et al., 1983). Briefly, exponentially growing cells were washed and resuspended in hypotonic medium (DMEM/water, 1:1). After 5 minutes at 37°C, they were washed in medium without $K^+$ (100 mM NaCl, 50 mM HEPES pH 7.4) and incubated at 37°C in the same medium with 1 mg/ml bovine serum albumin, with or without 10 mM KCl. After 25 minutes in these conditions, endocytosis was measured (see below). More than 80% of the cells were alive at the end of each experiment, as assessed by Trypan Blue exclusion.

### Cytosol acidification

Cytosol acidification was carried out as described (Sandvig et al., 1987). Briefly, the cells were washed and incubated for 30 minutes at 37°C in RPMI, 50 mM HEPES, 1 mg/ml bovine serum albumin, $NH_4Cl$, pH 7.2. The optimal concentration of $NH_4Cl$ varied somewhat depending on the cells and was 30 mM for IARC 301.5 and 40 mM for YT. After this incubation, the cells were centrifuged and resuspended in 140 mM KCl, 40 mM HEPES, 1 mg/ml bovine serum albumin, 1 mM amiloride (Sigma), pH 7.0, for 2 minutes at 37°C, in order to inhibit the $Na^+/H^+$ antiporter of cells loaded with $NH_4^+$. Thereafter, endocytosis of radiolabeled ligands was measured (see below). More than 80% of the cells were alive at the end of these experiments.

### Chlorpromazine treatment

IARC 301.5 cells were washed and incubated for 30 minutes at 37°C with 100 μM chlorpromazine (Sigma) in RPMI, 20 mM HEPES, 1 mg/ml bovine serum albumin, pH 7.2. Thereafter, endocytosis of radiolabeled ligands was measured (see below). These chlorpromazine concentrations and times of incubation were chosen so that cell viability was not affected.

### Labeling of reagents

Pure recombinant IL2 (SANOFI, France) and human transferrin (Sigma), loaded with iron (Ciechanover et al., 1983), were radiolabeled with $^{125}$iodine by the chloramine T method to specific activities of 360 μCi/μg and 20 nCi/μg, respectively. For labeling of IL2, three successive additions of chloramine T were performed within 5 minutes at room temperature to a final concentration of 25 μg/ml. The reaction was stopped after another 5 minutes, and labeled ligand was separated from free $^{125}$I by passage through an Excellulose GF-5 column (Pierce, Ill., USA). Ricin (a gift from Dr Patrice Boquet, Institut Pasteur, Paris) was labeled using IODO-GEN (Pierce, Ill., USA) to a specific activity of 2 μCi/μg as described (Sandvig et al., 1987).

Purified monoclonal antibodies were radiolabeled with $^{125}$I by the chloramine T method to specific activities of 0.2 μCi/μg for anti-transferrin receptor monoclonal antibody OKT9, and 10 μCi/μg for anti-IL2 receptor monoclonal antibodies 7G7B6 and 561.

### Receptor-mediated and fluid-phase endocytosis

After potassium depletion, cytosol acidification or chlorpromazine treatment, radiolabeled ligands were added to the cells at 37°C. The concentrations used were 10 nM for $^{125}$I-ferrotransferrin, 100 pM for $^{125}$I-IL2 (a concentration at which only high-affinity receptors are occupied), and 1 to 3 nM for $^{125}$I-labeled monoclonal antibodies.

After incubation at 37°C for the indicated times, the cells were rapidly cooled to 4°C and washed twice. Cell surface-associated radioactive ligand was then removed by two successive acid pH treatments (2 minutes at pH 2.8) at 4°C as previously described (Duprez et al., 1988). Nonspecific binding, measured for each ligand by adding a 100-fold excess of the same unlabeled ligand, was less than 5% in every case. The efficiency of removal of cell surface-associated ligands by acid pH washes was measured for each ligand and was more than 99%. The data presented here show specific binding and internalization. Potassium depletion, cytosol acidification and chlor-

promazine treatment did not modify either nonspecific binding or the efficiency of acid stripping.

Endocytosis of ricin was performed as already described for the other ligands by adding 8 nM $^{125}$I-ricin. The amount of internalized ricin was measured after washing the cells in 0.1 M lactose (Sandvig et al., 1987) for 30 minutes at 20°C, a treatment that removes 99% of surface bound $^{125}$I-ricin. Fluid-phase endocytosis was followed by measuring the uptake of horseradish peroxidase (HRP, Boehringer, Germany). After $K^+$ depletion, cells were incubated with 5 mg/ml HRP at 37°C for the indicated times. After cooling to 4°C, extracellular HRP was removed by five washings, and intracellular HRP was assayed after lysing the cells.

## RESULTS

### Internalization of IL2 in cells after potassium depletion

In order to study the endocytic pathway followed by IL2, we first deprived a human T cell line, IARC 301.5, of potassium. Cellular potassium depletion inhibits endocytosis via clathrin-coated pits (Larkin et al., 1983, 1986; Moya et al., 1985; Heuser and Anderson, 1989; van Deurs et al., 1989). The inhibition of endocytosis was measured by following the entry of transferrin, a well established marker of the coated pit pathway (Dautry-Varsat et al., 1983; Hopkins and Trowbridge, 1983; Klausner et al., 1983; Goldstein et al., 1985). IARC 301.5 cells express about 3000 high-affinity IL2 receptors and 150,000 transferrin receptors (Duprez et al., 1985). All experiments were run in parallel with IL2 and transferrin, and the data presented show specific binding and endocytosis. Under normal conditions, the rate of endocytosis of IL2 is two times slower than that of transferrin (3.5% $min^{-1}$ versus 7.5% $min^{-1}$) (Fig. 1). The association kinetics of IL2 to its high-affinity receptors are very rapid ($t_{1/2}$~ few seconds) (Lowenthal and Greene, 1987), so that when IL2 was added to cells at 37°C, receptor binding was not rate limiting, and the different kinetics of entry observed for IL2 and transferrin cannot be due to differences in binding kinetics to their respective receptors. Potassium depletion resulted in a strong inhibition (~80%) of transferrin endocytosis (Fig. 1a), as well as an increase of about 50% in the receptor number at the cell surface after 25 minutes without $K^+$ (not shown). Under such conditions, IL2 endocytosis was only partly inhibited (~50%) (Fig. 1b). Potassium depletion inhibited internalization via coated pits reversibly, since endocytosis of transferrin and IL2 was essentially restored when potassium was added back to depleted cells (Fig. 1c). The presence of IL2 did not modify the internalization of the transferrin receptor, and conversely (not shown). Experiments were also performed in which IL2 or transferrin were bound to cells at 4°C, excess ligand was washed away, and internalization was started by switching the cells to 37°C. The results are similar to those presented in Fig. 1 (not shown).

After binding to specific cell surface receptors, the toxin ricin is endocytosed in part by a clathrin-independent pathway (Moya et al., 1985; Sandvig et al., 1987). We therefore studied its internalization in IARC 301.5 cells. In potassium-depleted cells, ricin was still internalized at 45% of the value in control cells, in agreement with published data (not shown) (Moya et al., 1985; Sandvig et al., 1987). Finally, fluid-phase endocytosis, as measured with horseradish peroxidase, was only partially inhibited after potassium depletion (Fig. 1d), implying that non-clathrin-mediated endocytosis is responsible for at least half of total endocytosis in this T cell line.

### Internalization of IL2 receptors in potassium-depleted cells

To follow directly IL2 and transferrin receptor internalization, monoclonal antibodies directed against the IL2 and transferrin receptors were used as receptor ligands. Two anti-IL2 receptor monoclonal antibodies, directed against the α and β chains, were employed. These monoclonal antibodies do not affect the binding of IL2 and transferrin to their respective

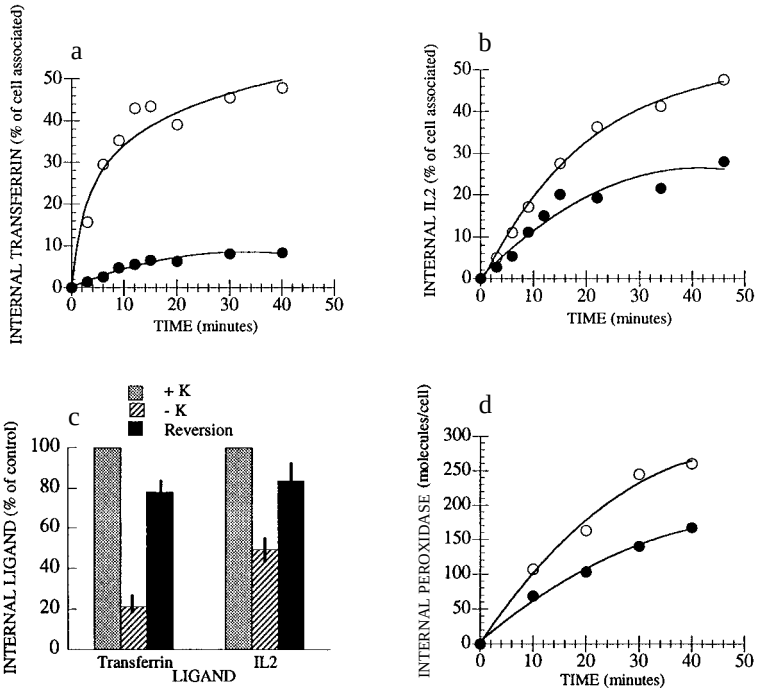


**Fig. 1.** Effect of potassium depletion on the internalization of transferrin, IL2 and HRP in IARC 301.5 cells. (a,b,d) Kinetics of endocytosis of $^{125}$I-transferrin (a), $^{125}$I-IL2 (b) or HRP (d) in cells that had been $K^+$-depleted (●) or not (○) after an osmotic shock, as described in Materials and Methods. (c) Restoration of endocytosis. After $K^+$ depletion, 10 mM KCl or control buffer were added at 37°C, and 10 minutes later, transferrin (left) or IL2 (right) were added. After further incubation for 15 minutes, the percentage of internalized ligand was measured (black), and compared with that in cells still $K^+$-depleted (hatched) or in control cells that had never been depleted (stippled). All experiments were done in triplicate.

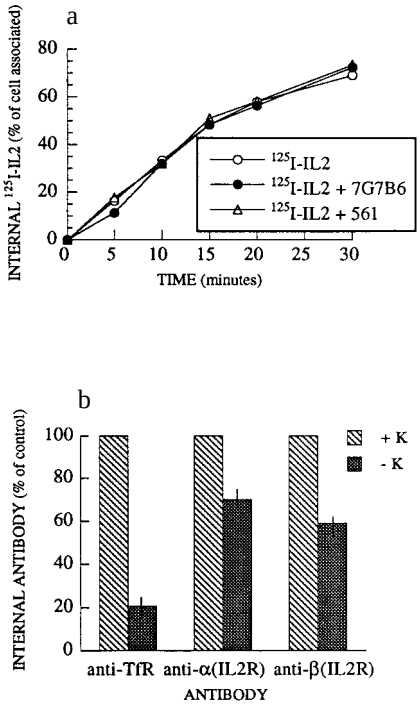


**Fig. 2.** Internalization of monoclonal antibodies as ligands for the transferrin and IL2 receptors. (a) Kinetics of $^{125}$I-IL2 endocytosis in the absence (○) or presence of anti-IL2 receptor monoclonal antibodies 7G7B6 (●) and 561 (△), performed in untreated cells, in RPMI, 20 mM HEPES, pH 7.2, 1 mg/ml bovine serum albumin. (b) Effect of $K^+$ depletion on monoclonal antibody internalization. The internalization of $^{125}$I-labeled monoclonal antibodies directed against the transferrin receptor, TfR (OKT9, left), or the IL2 receptor α chain (7G7B6, center) or β chain (561, right) with 200 pM IL2, was measured for 15 minutes with cells that had been osmotically shocked and $K^+$-depleted (stippled) or not (hatched), as described in Materials and Methods. In each case the percentage of internalized ligand in $K^+$-depleted cells is expressed relative to that in non-$K^+$-depleted cells.

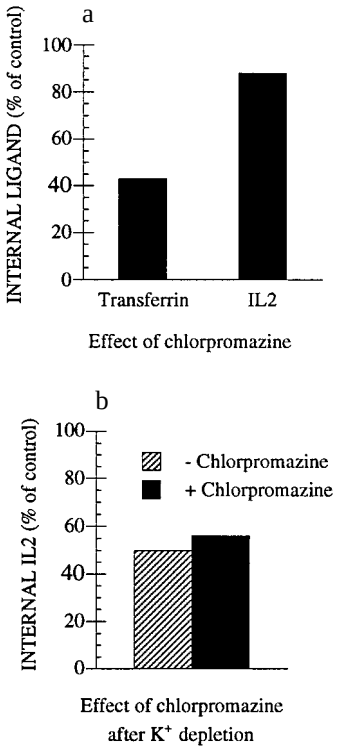


**Fig. 3.** Effect of chlorpromazine treatment on IL2 and transferrin internalization in IARC 301.5 cells. (a) After 30 minutes of incubation of the cells with 100 μM chlorpromazine, radiolabeled ligand was added and the cells were further incubated at 37°C. After 5 minutes and 10 minutes for transferrin and IL2, respectively, the amount of internalized ligand was measured. These incubation times were chosen so that they correspond to the linear part of the kinetics of entry of IL2 and transferrin. The percentage of internalized ligand in chlorpromazine-treated cells, is expressed relative to that in untreated cells. (b) Chlorpromazine treatment was performed on potassium-depleted cells. The percentage of internalized $^{125}$I-IL2 was measured after a 10 minute incubation at 37°C. The percentage of internalized IL2 in potassium-depleted cells, treated or not with chlorpromazine, is expressed relative to that in control, non-$K^+$-depleted, cells.

receptors, nor do they modify the kinetics of IL2 or transferrin entry (Weissman et al., 1986) (Fig. 2a). The endocytosis of radiolabeled monoclonal antibodies was receptor mediated and specific, since their internalization was prevented by an excess of the same unlabeled monoclonal antibody, and not by an excess of irrelevant monoclonal antibody. In IARC 301.5 cells deprived of potassium, the internalization of radiolabeled anti-IL2 receptor monoclonal antibodies was diminished by only 30-40%. However, the entry of anti-transferrin receptor monoclonal antibody was strongly inhibited (~80%) (Fig. 2b). In the experiments presented here, binding and internalization of anti-receptor monoclonal antibodies were measured in the presence of the receptor's natural ligand. The same result was obtained in the absence of IL2 and transferrin (not shown).

## Effect of chlorpromazine on IL2 internalization

It has been recently reported that cationic amphiphilic drugs such as chlorpromazine cause clathrin lattices to assemble on endosomal membranes and partially inhibit coated pit assembly at the plasma membrane (Wang et al., 1993). We have analyzed the effect of chlorpromazine on endocytosis in IARC 301.5 cells. The cells were treated with 100 μM chlorpromazine at 37°C before adding radiolabeled ligand. All experiments were run in parallel with IL2 and transferrin. This treatment marginally affected IL2 endocytosis, while transferrin entry was inhibited by 60% (Fig. 3a).

Next we asked if the potassium depletion-resistant entry of IL2 described above was modified by chlorpromazine treatment, which affects clathrin-coated pit entry by a mechanism different from potassium depletion. Potassium depletion was performed as described in Materials and Methods, except that 100 μM chlorpromazine was added to the medium without potassium for 30 minutes. As shown in Fig. 3b, chlorpromazine did not affect IL2 internalization in potassium-depleted cells.

## Internalization of IL2 in cells after cytosol acidification

The extent of inhibition of endocytosis via coated pits by

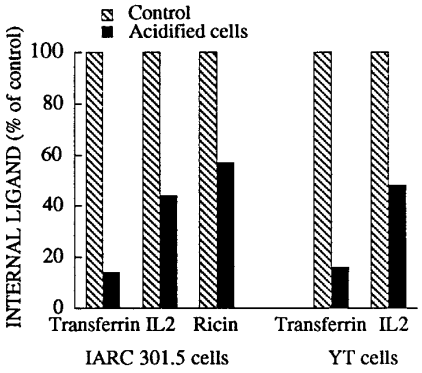


**Fig. 4.** Effect of cytosol acidification on the internalization of transferrin, IL2 and ricin in IARC 301.5 and YT cells. After cytosol acidification (black) or not (hatched), radiolabeled ligand was added and the cells were further incubated at 37°C. After 3 minutes, 10 minutes and 15 minutes for transferrin, IL2 and ricin, respectively, the amount of internalized ligand was measured. In each case the percentage of internalized ligand in cytosol-acidified cells is expressed relative to that in untreated cells.

potassium depletion varies depending on the cell type (Moya et al., 1985; van Deurs et al., 1989). Another inhibition method has been used that is efficient in most cell lines and consists of acidification of the cell cytosol. This approach also has the advantage of relying on a different mechanism of inhibition (Sandvig et al., 1987; Heuser, 1989). Cytosol acidification was performed on IARC 301.5 and activated YT cells. The cells were acidified by the ammonium chloride/amiloride method described by Sandvig et al. (1987). In control and acidified cells, endocytosis of transferrin, IL2 or ricin was carried out for 3, 10 or 15 minutes, respectively. These incubation times were chosen so that the kinetics of entry for each ligand are within the linear range (see Fig. 1). All experiments with IL2 and ricin were run in parallel with transferrin. Moreover, the presence of IL2 did not modifiy transferrin endocytosis, and conversely. In both cells, cytosol acidification resulted in 85% inhibition of transferrin endocytosis, while IL2 endocytosis was inhibited by only 50% (Fig. 4).

Ricin internalization was also analyzed in cytosol-acidified T lymphocytes, since it has been shown that it is still internalized after cytosol acidification of other cell types (Sandvig et al., 1987). In acidified IARC 301.5, the entry of ricin was also inhibited by only 45% (Fig. 4).

Several combinations of IL2 receptor chains are known that form high-, intermediate- or low-affinity receptors for the ligand IL2. Both high-affinity receptors (composed of the α, β and γ chains) and intermediate-affinity receptors (composed of β and γ) internalize IL2 and transduce IL2 signaling (Smith, 1989). A subclone of YT cells, YT 2C2, expresses intermediate- but not high-affinity receptors (Teshigawara et al., 1987). After cytosol acidification, these cells internalize both $^{125}$I-IL2 and $^{125}$I-anti-IL2 receptor β chain monoclonal antibody to about 55% of the value obtained in control cells, while transferrin endocytosis is inhibited to 15% of the value in control cells (not shown). Thus IL2 is still internalized in cells in which clathrin-dependent endocytosis is inhibited, whether it is bound to high- or to intermediate-affinity receptors.

## DISCUSSION

Several methods have been described for inhibiting endocytosis via coated pits. Cellular potassium depletion and cytosol acidification have been shown to prevent internalization through clathrin-coated structures, but not by alternative routes. Treatment with the cationic amphiphilic drug chlorpromazine results in a redistribution of AP-2 to intracellular compartments by a mechanism that is not fully understood. In this work, we have subjected human T lymphocytes and natural killer cells expressing IL2 receptors to these various treatments. As expected, potassium depletion and cytosol acidification resulted in a strong decrease of transferrin internalization. However, IL2 receptors were still efficiently internalized, to about 50% of control levels, as probed with iodinated IL2 or monoclonal antibodies. The extent of IL2 receptor internalization was roughly the same, regardless of the inhibition mechanism or cell type used. Chlorpromazine was used as an agent that disrupts AP-2 traffic and coated-pit endocytosis. The conditions of chlorpromazine treatment chosen did not affect cell viability and resulted in a 60% inhibition of transferrin endocytosis. IL2 endocytosis was barely inhibited by this treatment. Furthermore, in potassium-depleted cells, the remaining IL2 endocytic activity (50% of control levels in untreated cells) was unaffected by chlorpromazine treatment.

Taken together, these results can be interpreted in two ways: either IL2 and its receptors enter cells by dual pathways, the coated pit one and another one, or they enter solely via coated pits. In the latter case, our results would imply that the few coated pits that remain functional after potassium depletion or cytosol acidification are sufficient to internalize 50% of IL2 receptors, while less than 20% of transferrin receptors are internalized. This would suggest that IL2 receptors compete better than transferrin receptors for coated pits that resist $K^+$ depletion or cytosol acidification. This possibility cannot be excluded, although it seems unlikely in view of the faster kinetics of entry of transferrin as compared to IL2, which suggest that transferrin receptors have a better affinity than IL2 receptors for the endocytic machinery. In addition, the fact that chlorpromazine treatment did not affect IL2 internalization after potassium depletion does not favor this hypothesis. The other possibility is that IL2 receptors can enter cells in part by a process that does not involve clathrin-coated pits. Other ligands have been reported to enter cells by dual pathways. The toxin ricin binds to galactosyl residues on a variety of surface glycoproteins and glycolipids. Its rate of entry is low, with about 8% of total surface-bound ligands internalized within 10 minutes (Moya et al., 1985; Sandvig et al., 1987), and endocytosis is only partly inhibited when the clathrin-coated pit pathway is blocked. Electron microscopy has shown that ricin enters via uncoated stuctures (Sandvig et al., 1987). The EGF and insulin receptors, both members of the tyrosine kinase receptor family, may also use two pathways of entry. One has been described as rapid and is referred to as the clathrin-coated one, while the other one appears to be about five times slower and does not seem to depend on clathrin (Lund et al., 1990; Backer et al., 1991). Based on the partial inhibition of IL2 endocytosis found when clathrin-coated entry was prevented, it appears that IL2 may also use in part the clathrin-coated pit pathway, in agreement with the fact that some IL2 has been found in coated pits by electron microscopy (McCrae et al.,

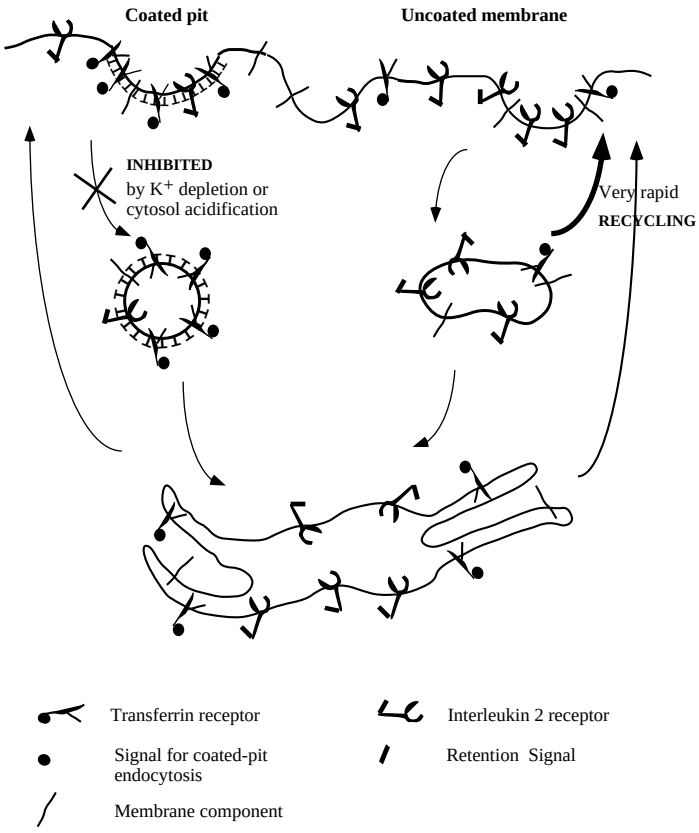


**Fig. 5.** A model of endocytosis. See text for details.

1988; Peters and Norback, 1990). The alternative pathway of entry of IL2 receptors has been detected in cells where coated pit internalization was inhibited, and therefore we cannot rule out that, under normal conditions, this may represent a minor way for endocytosis.

During the past decade, the term endocytosis has become virtually synonymous with internalization via clathrin-coated pits and vesicles. However, as discussed above, many lines of evidence point to the existence of non-clathrin-mediated internalization mechanisms. From these data and our own results concerning IL2 receptors, we would like to propose a speculative model on how endocytosis may proceed (Fig. 5). Rapid and constant endocytosis is a characteristic of the plasma membrane. For most membrane components, it is followed by very rapid and constitutive recycling (Steinman et al., 1983; Mayor et al., 1993). The internalization measured for any molecule is the net result of its endocytosis and recycling. Two types of signals can interfere with this very rapid membrane movement at different endocytic steps. (i) Some membrane proteins carry a signal for clathrin-coated pit localization (reviewed by Trowbridge, 1991) and cluster in the pits to an extent depending on their affinity for the underlying adaptors (Pearse and Robinson, 1990). We propose that membrane components may recycle from uncoated structures, but not from coated vesicles. The difference in efficiency between endocytosis by uncoated membrane invagination and clathrin-coated pit endocytosis could therefore be due to slower recycling in the latter case. An alternative, compatible with this one, would be that internalization may simply be faster via coated pits. (ii) Many lines of evidence suggest that some membrane proteins carry a retention signal that presumably prevents their rapid recycling from intracellular uncoated endocytic compartments (Hopkins, 1992). This hypothetical retention signal may also be the same as the degradation signal that directs some receptors towards intracellular degradation. In this model, if a receptor does not have a strong coated-pit internalization signal, but carries a retention signal, it would be internalized by membrane bulk flow but would escape rapid constitutive recycling. It would be efficiently internalized, even in the absence of functional coated pits, and would remain in intracellular compartments before being degraded. We propose that IL2 receptors undergo this process.

Under conditions where entry through coated pits is impaired, membrane bulk flow endocytosis could still occur through uncoated membrane invaginations. However, receptors carrying an internalization signal via coated pits (Trowbridge, 1991), such as transferrin receptors, would then be mostly trapped because of their interaction with adaptors, which are still present on the plasma membrane after potassium depletion or acidification (Hansen et al., 1993a; Sorkin and Carpenter, 1993). Therefore, they would not be available for the non-coated process. Assuming that they could still enter through uncoated areas of the membrane, they would recycle very rapidly, as they do not carry a retention signal. Our experiments indicate that, under normal conditions, IL2 is internalized about two times more slowly than transferrin. IL2 receptors may lack a strong endocytic signal, and therefore mostly use an uncoated pathway. Hence, disruption of the pits would not much affect their efficiency of entry. Comparing the internalization of IL2 in potassium-depleted cells with that of transferrin in control cells, it appears that entry via the uncoated pathway is at most five to six times slower than via coated pits. However, this estimate relies on experiments performed in potassium-depleted cells, when some IL2 receptors may be trapped in coated structures and endocytosis via uncoated structures may be slower because of the depletion. Moreover, IL2 receptors may partly recycle, if their retention signal is not very strong. For all these reasons, the kinetics of entry via uncoated invaginations may be underestimated and may in fact not be significantly slower than that via coated structures.

In the model presented in Fig. 5, we have assumed that both internalization pathways rejoin in an early endocytic organelle, in agreement with published reports (Tran et al., 1987; Hansen et al., 1993b) and our own results (unpublished).

We think that our model may reconcile data obtained by different groups on clathrin-independent receptor-mediated endocytosis. EGF receptors, for instance, may be endocytosed by both coated and uncoated areas of the plasma membrane, as has already been proposed (Lund et al., 1990). Binding of EGF increases the affinity of its receptors for coated pits (Sorkin and Carpenter, 1993) and favors their endocytosis through the coated pathway. Moreover, it activates the tyrosine kinase of the receptors (Ushiro and Cohen, 1980), triggering a signal, presumably a retention signal, that leads to further degradation of the receptor-ligand complexes (Carpenter and Cohen, 1979). Indeed, kinase-negative EGF receptors are endocytosed as wild-type receptors, but rapidly return to the

cell surface without being degraded (although they were initially reported not to be internalized; Honegger et al., 1987; Felder et al., 1990). Thus, internalization of EGF receptors is induced by ligand binding, both by increasing their affinity for coated pits and by preventing their recycling. Similar results have been reported for the insulin receptor (Backer et al., 1991).

A variation of this model proposes that the vesicular process that involves clathrin coats simply continues when they are removed. In this case, the internalization efficiency of a receptor would depend on the possibility of its intracellular retention as discussed above.

An alternative to our model is that, as previously proposed, there exist other endocytic pathways, each with their own specific markers (reviewed by Watts and Marsh, 1992). However, our model can account for our data and published results without having to postulate the existence of specific, alternative pathways to the clathrin-coated pit one. The possibility that non-clathrin-coated internalization may be a minor pathway under normal conditions and induced when clathrin-coated pits are not functional cannot be ruled out. However, in the case of EGF receptors, clathrin-independent internalization has also been observed when the clathrin-coated pit pathway is functional (Lund et al., 1990). Internalization via clathrin-coated pits has been documented mostly by electron microscopy studies. It would be of interest to use IL2 to observe clathrin-independent endocytosis. However, this may prove difficult, since even transferrin receptors, which are internalized through coated pits, are localized mostly outside coated pits at steady state (10% in coated pits) (Miller et al., 1991; Hansen et al., 1992). If one turns to intracellular vesicles, one would expect to find IL2 in both coated and uncoated vesicles, and it will be difficult to distinguish between a smooth vesicle and a vesicle having simply lost its clathrin coat.

Another entry route into cells, through caveolae, has recently been described and named potocytosis (Anderson et al., 1992; Rothberg et al., 1992). It has been suggested that caveolae might also mediate endocytosis (Dupree et al., 1993; van Deurs et al., 1993). Caveolin, a major component of caveolae, could not be detected in T lymphocytes, using an anti-caveolin monoclonal antibody (not shown). In addition, potocytosis does not involve formation of vesicles, while IL2 receptors are found in intracellular compartments (Ferrer et al., 1993). These observations do not support the possibility that caveolae may be involved in endocytosis of IL2.

In conclusion, we show that IL2 receptor endocytosis may occur even in the absence of functional clathrin-coated pits. It remains to be seen whether this also turns out to be the case for endocytosis of other receptors of the cytokine receptor family. Finally, it will be interesting to determine whether this endocytic mechanism, operating alongside the clathrin-coated one, is essential for some cell functions.

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