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The internalization signal and the phosphorylation site of transferrin receptor are distinct from the main basolateral sorting information

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Wild-type human transferrin receptor (hTfR), like endogenous canine receptor, is expressed almost exclusively (97%) at the basolateral membrane of transfected Madin-Darbey canine kidney (MDCK) cells. We investigated the role of two distinct features of the hTfR cytoplasmic domain, namely the endocytic signal and the unique phosphorylation site, in polarized cell surface delivery. Basolateral location was not altered by point mutation of Ser24→Ala24, indicating that phosphorylation is not involved in vectorial sorting of hTfR. The steady state distribution of hTfR was partially affected by a deletion of 36 cytoplasmic residues encompassing the internalization sequence. However, 80% of the receptors were still basolateral. As assessed by pulse-chase experiments in combination with biotinylation, newly synthesized wild-type and deletion mutant receptors were directly sorted to the domain of their steady state residency. Although both receptors could bind human transferrin, endocytosis of the deletion mutant was strongly impaired at either surface. These data indicate that the predominant basolateral targeting signal of hTfR is independent of the internalization sequence.

Key words: basolateral sorting/endocytosis/human transferrin receptor/MDCK cells

Introduction

The plasma membrane of epithelial cells is divided by tight junctions into an apical and basolateral domain, each with a distinct protein and lipid composition. This asymmetrical distribution of membrane components enables epithelial cells to perform specialized vectorial transport functions. Elucidation of the mechanisms whereby specific proteins and lipids are accurately targeted to either plasma membrane domain is of central importance to the understanding of cell polarity (Rodriguez-Boulan and Nelson, 1989; Simons and Wandinger-Ness, 1990; Hopkins, 1991; Mostov *et al.*, 1992).

From studies performed in different epithelial cells such as enterocytes (Massey *et al.*, 1987; Le Bivic *et al.*, 1990a; Matter *et al.*, 1990), hepatocytes (Bartles *et al.*, 1987), thyroid (Zurzulo *et al.*, 1992) or tubular kidney cells (Caplan *et al.*, 1986; Brändli *et al.*, 1990; Le Bivic *et al.*, 1990b; Simons and Wandinger-Ness, 1990) two major sorting sites for the membrane proteins have been defined, the *trans*-Golgi network (TGN) and the basolateral membrane. Depending on the cell type and protein, newly made proteins travel directly from the TGN to the apical or basolateral domain or indirectly to the basolateral membrane from where they are redirected to the apical surface.

Regardless of the pathway taken by plasma membrane proteins, sorting and selective transport in epithelial cells imply specific interactions with membrane and/or cytosolic components. A molecular signal might serve as a common recognition structure for membrane proteins with the same destination. Results with chimeric viral glycoproteins suggested that this information resides within the transmembrane and ectodomains (McQueen *et al.*, 1986, 1987; Roman and Garoff, 1986; Roth *et al.*, 1987; Compton *et al.*, 1989). The first clearly established signal for polarized sorting was the glycosyl-phosphatidylinositol anchor which directs proteins to the apical membrane (Brown *et al.*, 1989; Lisanti *et al.*, 1989). Recent studies performed with poly-Ig receptor (Mostov *et al.*, 1986; Casanova *et al.*, 1991), natural isoforms of the Fc receptor type II or LDL receptor (Hunziker *et al.*, 1991) have shown that signals required for basolateral delivery are contained within the cytoplasmic domain. This information can be classified into two groups: those that overlap with the endocytic signal (Fc receptor type II, lysosomal protein gp120, hemagglutinin, human nerve growth factor receptor) (Brewer and Roth, 1991; Hunziker *et al.*, 1991; Le Bivic *et al.*, 1991) and those that are separate from such a determinant (LDL receptor, poly-Ig receptor) (Casanova *et al.*, 1991; Hunziker *et al.*, 1991; Yokode *et al.*, 1992).

Transferrin receptor (TfR) mediates cellular uptake by internalizing and recycling the serum iron transport transferrin (Tf). The receptor is a transmembrane glycoprotein composed of two identical subunits of 95 kDa linked by disulfide bridges (McClelland *et al.*, 1984; Schneider *et al.*, 1984). In Madin-Darbey canine kidney (MDCK) cells, TfR is expressed exclusively at the basolateral surface (Fuller and Simons, 1986). The polarized distribution is maintained by accurate recycling to this membrane domain following endocytosis. It has been shown previously that the cytoplasmic domain and more precisely the YTRF motif is necessary for sorting of TfR into coated pits prior to endocytosis (Rothenberger *et al.*, 1987; Iacopetta *et al.*, 1988; Collawn *et al.*, 1990; Jing *et al.*, 1990). In the present report, we investigated whether the endocytic signal or phosphorylation of the cytoplasmic domain are required for the polarized expression of human transferrin receptor (hTfR) in epithelial cells. When transfected in MDCK cells, the wild-type receptor was targeted efficiently to the basolateral membrane where it

bound and endocytosed Tf. A mutation of the phosphorylation site did not affect this polarized distribution. On the other hand, hTfR with a cytoplasmic deletion of 36 amino acids encompassing the endocytosis motif YTRF and thus unable to endocytose Tf was delivered with 80% efficiency from the TGN to the basolateral membrane.

Results

Expression of wild-type and mutant hTfR in MDCK cells

In order to test whether the endocytic signal or the phosphorylation site on serine 24 are required for the strongly polarized expression of TfR at the basolateral surface of epithelial cells, we transfected previously described hTfR cDNA clones (Rothenberger *et al.*, 1987) into MDCK type I cells (Fuller *et al.*, 1984). The plasmid constructs are derived from the full-length hTfR cDNA clone pcD-TR1 (Kühn *et al.*, 1984) and are transcribed from the genomic hTfR promoter (Owen and Kühn, 1987). They lack the 3′ untranslated sequences known to reduce receptor expression due to feedback regulation by iron (Müllner and Kühn, 1988). The expression vectors code either for wild-type hTfR, a point mutant at the serine 24 phosphorylation site or a deletion mutant that lacks amino acids 6–41 in the cytoplasmic domain (Rothenberger *et al.*, 1987; Figure 1). Transfection of MDCK cells with these vectors was achieved by calcium phosphate precipitation, and the hygromycin B resistance gene was used as a selectable marker. Cells expressing high levels of hTfR at their cell surface were isolated from hygromycin B resistant populations by sorting on a fluorescence-activated cell sorter (FACS) after having been stained with anti-hTfR antibody. These cells were subcloned to obtain stable cell lines and four of them were selected for the study. They express wild-type (K7.2), deletion mutant (K12.4 and K12.6) or phosphorylation site mutant (K12.3) forms of hTfR (Figure 1 and Table I). The high electrical resistance measured 5 days after seeding cells on to nucleopore filters indicated that transfected as well as parental MDCK monolayers established tight junctions (Table I). This was directly confirmed by electron microscopy (data not shown).

To estimate the level of hTfR expression in selected clones, cells were labelled overnight with [^{35}S]methionine/[^{35}S] cysteine (Tran^{35}S label). Human TfR was immunoprecipitated using a monoclonal anti-hTfR antibody B3/25 and analysed by SDS–PAGE and fluorography (Figure 2). No TfR was immunoprecipitated from the non-transfected MDCK cells demonstrating that the monoclonal antibody does not cross-react with canine TfR. K7.2 and K12.3 cells expressed a 95 kDa hTfR, whereas a slightly smaller protein of 91 kDa was immunoprecipitated from K12.4 and K12.6 cells tranfected with the hTfR deletion mutant. K7.2 and K12.4 cells had the strongest level of hTfR expression, being ~5-fold higher than in the K12.3 and K12.6 cell lines. No canine TfR was co-precipitated by the antibody (Figure 2) indicating that transfected hTfR did not form covalent heterodimers with endogenous TfR.

Fig. 1. Amino acid sequence of the cytoplasmic domain of hTfR. hTfR is a transmembrane protein with a cytoplasmic N-terminal domain of 65 amino acids followed by its membrane domain (grey box). The deletion of residues 6–41 used in this study is represented by the black box and the unique phosphorylation site Ser24 is indicated by a star.

Polarized expression of wild-type and mutant hTfR in MDCK cells

We investigated the cell surface distribution of the different forms of hTfR transfected in MDCK cells by using a biotin assay (Sargiacomo *et al.*, 1989). MDCK monolayers grown on nucleopore filters were labelled with sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin) from the apical or basolateral side. hTfR was immunoprecipitated with

Table I. MDCK cell lines transfected with wild-type or mutated versions of hTfR cDNA

Transfected hTfR cDNA	Cell line	Resistance ($\Omega \cdot cm^2$)
None	MDCK	5520
Wild-type	K7.2	6640
Deletion mutant Δ6–41	K12.4	5120
Deletion mutant Δ6–41	K12.6	6550
Point mutant Ser24→Ala24	K12.3	3200

Cells were seeded at confluent density on Costar nucleopore filters. Trans-epithelial electrical resistance was measured 5 days later. Values represent the average of four experiments.

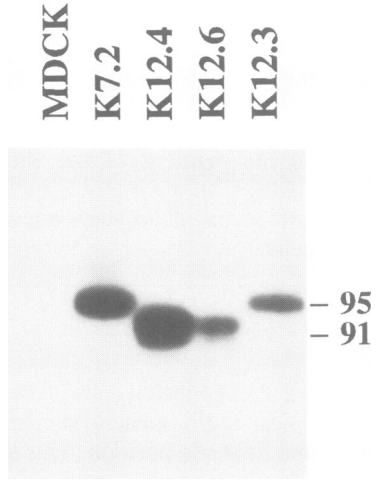

Fig. 2. Expression of hTfR mutants in MDCK cells. Non-transfected MDCK cells or transfected MDCK cell clones expressing different hTfR mutants were labelled overnight with 0.5 mCi/ml of ^{35}S-labelled methionine and cysteine as described in Materials and methods. hTfR was immunoprecipitated and analysed on a 6–15% SDS–polyacrylamide gel followed by fluorography. The molecular mass of 95 kDa indicates the migration of wild-type hTfR (in cell clone K7.2) and serine 24 phosphorylation site mutant hTfR (clone K12.3), whereas 91 kDa corresponds to the cytoplasmic deletion mutant hTfR (in clones K12.4 and K12.6).

B3/25 antibody and biotinylated hTfR revealed after SDS−PAGE by [^{125}I]streptavidin blotting (Figure 3). This experiment showed that 97% of wild-type hTfR was localized to the basolateral membrane (Figure 3 and Table II), also known to be the site of expression of endogenous canine TfR (Fuller and Simons, 1986). The basolateral location of hTfR was not altered in the Ser24→Ala24 point mutant (Figure 3 and Table II) which lacks the unique cytoplasmic phosphorylation site on hTfR (Davis *et al.*, 1986; Rothenberger *et al.*, 1987). In contrast, 20% of the deletion mutant hTfR could be immunoprecipitated from the apical cell surface in two separate transfected cell clones, K12.4 and K12.6 (Figure 3 and Table II). To ensure that the introduction of plasmids and the selection for hTfR had not altered the polarized distribution of endogenous MDCK proteins, a major apical sialoglycoprotein, Gp114 (Le Bivic *et al.*, 1990b), was checked in the same biotin assay. It was found to be predominantly apical in all transfected MDCK cell clones (Figure 3 and Table II). This indicated that differences in the distribution of wild-type or deletion mutant hTfR were not due to intrinsic changes in the polarization of transfected MDCK cell lines.

In order to confirm these biochemical data, receptor localization was also investigated by indirect fluorescence

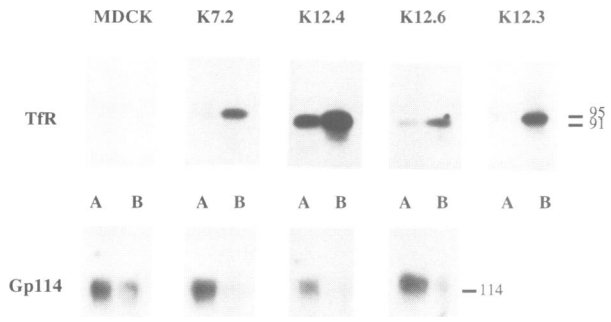

Fig. 3. Polarized surface distribution of wild-type or deletion mutant hTfR and of Gp114. The hTfR and MDCK glycoprotein Gp114 was immunoprecipitated after labelling of the apical (**A**) and basolateral (**B**) sides of MDCK cells with NHS-LC-Biotin. The biotinylated proteins were revealed after SDS−PAGE on a 6−15% gel by transfer to nitrocellulose and [^{125}I]streptavidin blotting. The apparent molecular mass of analysed proteins is indicated in kDa.

Table II. Polarized expression of hTfR and Gp114 in transfected MDCK cells

Cell line	Domain	hTfR (%)	Gp114 (%)
MDCK	apical	0	63
	basolateral	0	37
K7.2	apical	3	96.5
	basolateral	97	3.5
K12.4	apical	22	86
	basolateral	78	14
K12.6	apical	17.5	88
	basolateral	82.5	12
K12.3	apical	2.5	nd
	basolateral	97.5	nd

Autoradiographs from Figure 2 were quantitated by densitometry. The fraction of hTfR and canine Gp114 at the apical or basolateral domain was calculated as percentage of total on both domains. nd: not determined.

microscopy. K7.2 and K12.4 MDCK cells were grown on transparent nucleopore filters, fixed with formaldehyde, permeabilized with saponin and incubated with the anti-hTfR antibody B3/25 followed by a rhodamine-conjugated goat anti-mouse IgG antibody. The immunofluorescence patterns were analysed using a confocal laser scanning microscope. As shown in Figure 4A, both K7.2 and K12.4 cells expressed hTfR at the basal and lateral membranes. In contrast, only K12.4 cells exhibited staining at the apical surface. It should be noticed that although all the cells were labelled, the intensity of immunostaining of K12.4 cells was quite heterogeneous both at basolateral and apical membranes. Intracellular vesicular structures, probably endosomal, are labelled by the anti-hTfR antibody in K7.2 but not in K12.4 cells. Non-transfected MDCK cells did not show any fluorescence signal (data not shown).

We also examined the apical expression of hTfR by electron microscopy after immunogold labelling with the anti-hTfR antibody B3/25 and protein A−gold on living cells at 4°C (Figure 4B). Non-specific binding to the apical surface, as measured by the frequency of gold particles bound to MDCK cells, was ~30 grains/mm of cell surface (not shown). Transfectants expressing wild-type (Figure 4B) or phosphorylation site mutant hTfR (not shown) showed similarly low levels of labelling. In contrast, transfectants expressing deletion mutant hTfR were heavily labelled at their apical surface with 2090 and 11 978 gold particles/mm of sectioned cell surface in K12.6 and K12.4, respectively. These gold particles were particularly frequent over microvilli (Figure 4B).

It appears from these data that serine phosphorylation does not play a role in determining polarized expression of TfR in MDCK cells. Furthermore, deletion of residues 6−41 resulted in the expression of 80% of the truncated receptor at the basolateral membrane and 20% at the apical surface. This ectopic expression of a minor population of deletion mutant hTfR could be caused either by inefficient sorting from the TGN to the basolateral surface or by a default in the maintenance of hTfR basolaterally.

Binding and internalization of FITC-conjugated transferrin in hTfR transfected MDCK cells

It has been shown previously in L cells (Rothenberger *et al.*, 1987), in chicken embryonic fibroblasts (Jing *et al.*, 1990) and in CHO cells (Gironès *et al.*, 1991) that wild-type and truncated hTfR have the same affinity for hTf but that the endocytic capacity of the deletion mutant is dramatically reduced. In order to rule out the possibility of basolateral sorting followed by transcytosis, we verified whether wild-type and deletion mutant hTfR displayed the same properties in MDCK cells.

Both before and after EGTA treatment of cells grown on plastic dishes, the K_d for [^{125}I]Tf binding, calculated from Scatchard analysis, did not significantly differ between MDCK cell lines transfected with wild-type (K_d = 5.8×10^{-9} M) or deletion mutant hTfR (K_d = 10.2×10^{-9} M) (data not shown). To visualize apical and basolateral binding capacities, K7.2 and K12.4 cells were grown on cover-glasses, incubated at 4°C with FITC-conjugated human Tf for 30 min, fixed in formaldehyde and viewed on a confocal microscope. The intensity of the laser was set at a level that does not permit the detection of endogenous receptors. As shown in Figure 5 (upper panel), both wild-type and truncated hTfRs bound Tf at their respective

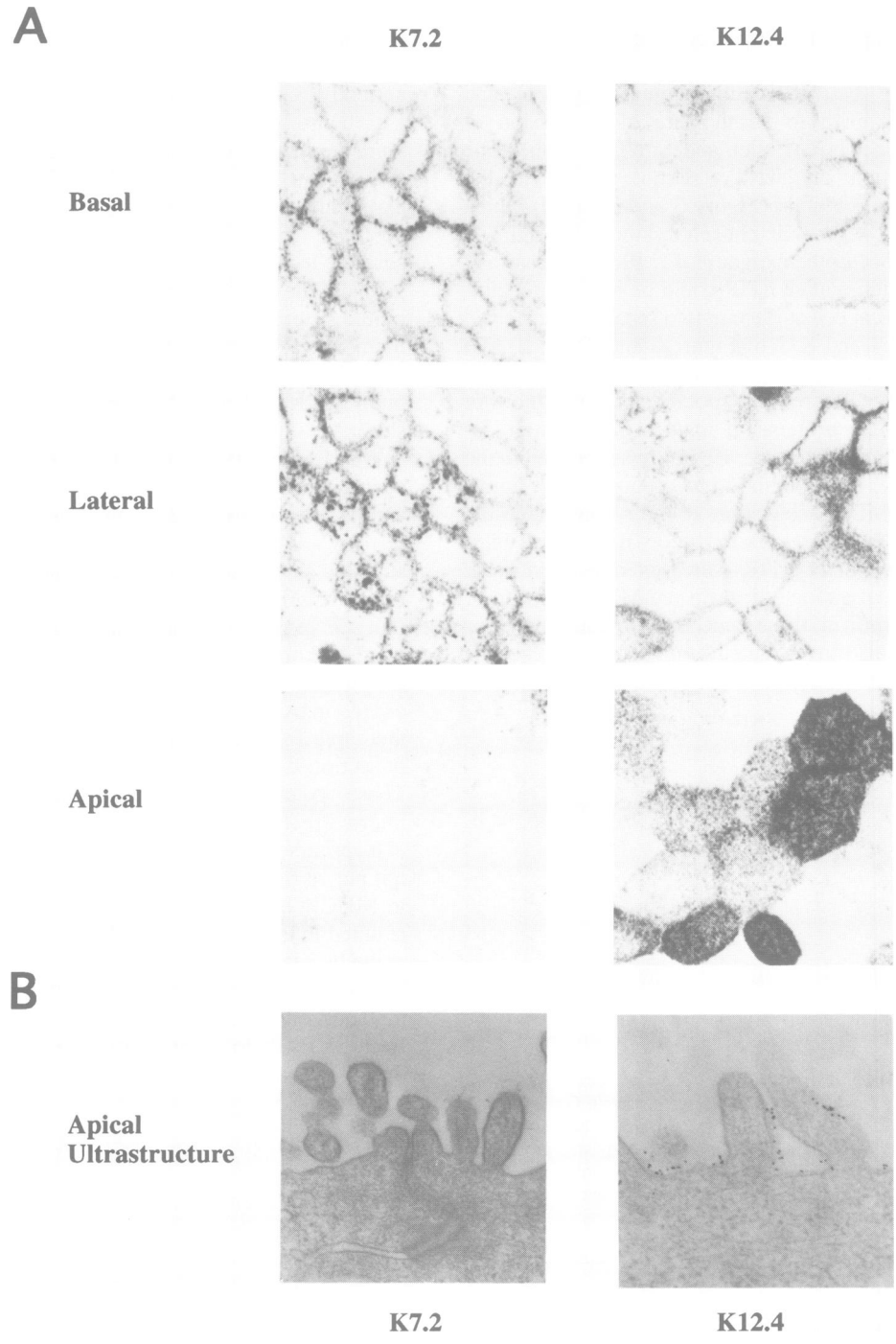

Fig. 4. Localization of wild-type and deletion mutant hTfR in MDCK cells. (**A**) Immunofluorescence staining of hTfR in permeabilized MDCK cells. Cells were grown on transparent nucleopore filters and fixed with 2% paraformaldehyde 5 days after confluency. Cells were then permeabilized in the presence of 0.075% saponin and incubated with the mouse anti-hTfR monoclonal antibody B3/25 followed by a rhodamine-conjugated goat anti-mouse Ig antibody. Each cell line was viewed under identical conditions with a confocal laser scanning microscope. A basal, lateral and apical picture was taken from sections spaced by 2 μm. (**B**) Immunogold staining of hTfR at the apical surface of transfected MDCK cell lines. Cells grown on nucleopore filters were incubated at 4°C with anti-hTfR antibody B3/25 followed by 5 nm protein A-coated gold particles and processed for electron microscopy as described in Materials and methods. Bars: 0.1 μm.

expression domains. To induce the internalization of Tf–hTfR complexes, cells were washed after Tf binding and incubated at 37°C for 10 min prior to fixation. In K7.2 cells, as expected, all basolateral Tf bound to wild-type receptors was internalized, whereas no intracellular labelling could be detected apically. In contrast, the truncated hTfR in K12.4 cells was unable to mediate any Tf internalization either at basolateral or apical membrane (Figure 5, lower panel). These results confirmed the intracellular distribution of hTfR detected by indirect immunofluorescence in Figure 4. The inability of deletion mutant hTfR to be internalized in MDCK cells favoured the hypothesis of a direct targeting of hTfR from the TGN to the basolateral and apical domains.

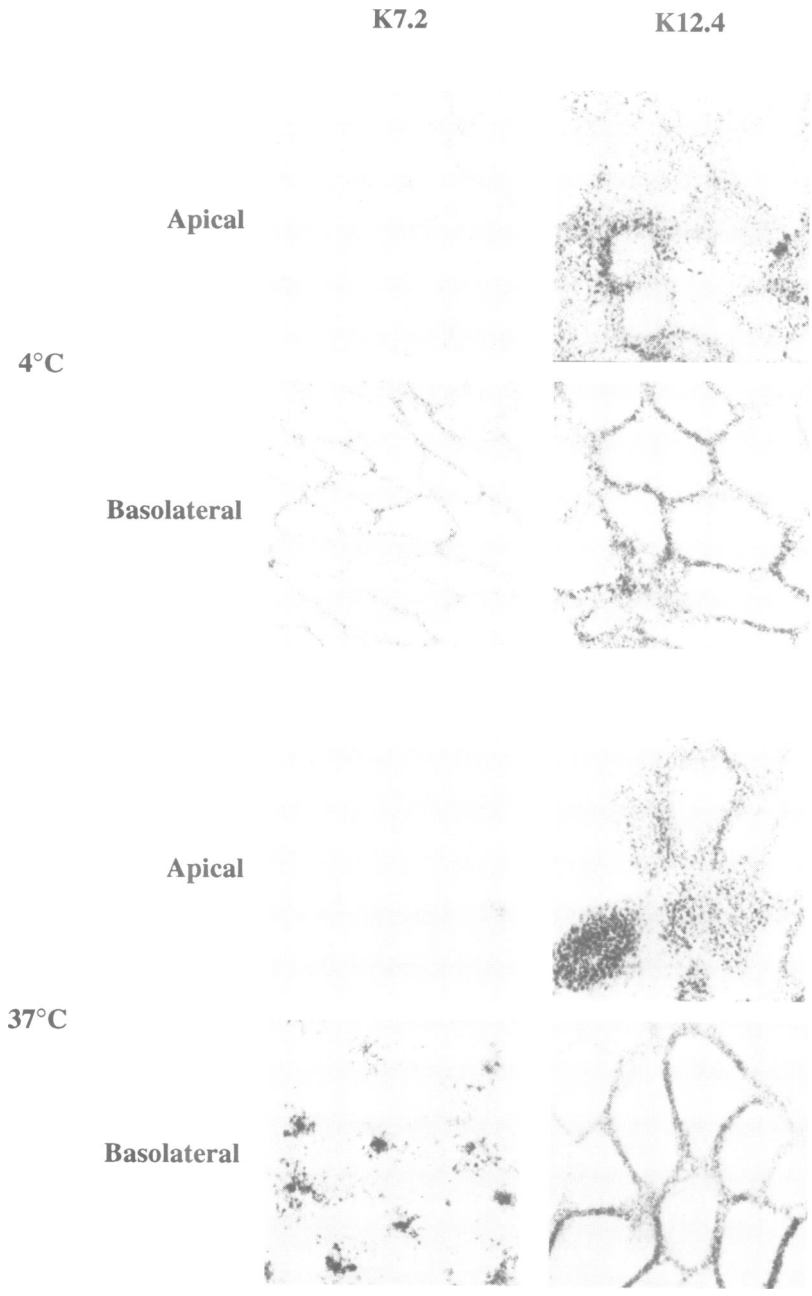

Fig. 5. Binding and internalization of FITC-conjugated transferrin in hTfR-transfected MDCK cells. MDCK cells grown on coverslips for 5 days were incubated with 60 nM FITC-Tf at 4°C for 30 min (upper panel), washed and then incubated at 37°C for 10 min (lower panel). Cells were fixed in 2% paraformaldehyde and viewed with a confocal laser scanning microscope. The threshold of fluorescence signal detection was adjusted so as to suppress the weak contribution of FITC-Tf-labelled endogenous TfR.

Processing and cell surface appearance of wild-type and deletion mutant hTfR in MDCK cells

To confirm the hypothesis of a direct sorting of hTfR from the intracellular sites to the surface, we compared the processing and routing of newly synthesized wild-type and deletion mutant hTfR in MDCK cells. The acquisition of complex carbohydrate chains was analysed after a 15 min metabolic pulse with ^{35}S-labelled methionine and cysteine followed by a chase with unlabelled medium. At different times, hTfR was immunoprecipitated with the B3/25 antibody and resolved by SDS−PAGE. An expected shift in the apparent molecular weight due to glycosylation was observed for both wild-type and deletion mutant hTfR (Figure 6A). The maturation of wild-type receptors was achieved within 1 h, while the hTfR deletion mutant was delayed in its processing by 90 min (Figure 6A and C). This prolonged retention of the cytoplasmic deletion mutant is probably due to a slower processing in the endoplasmic reticulum or *cis*-Golgi, since the acquisition of Endo H-resistant sites required 60 min instead of the 20 min observed for the wild-type receptor (Figure 6A). However, dimerization of the mutant hTfR occurred with the same kinetics as for the wild-type receptor (30 min; data not shown).

The surface delivery of wild-type and deletion mutant hTfR in cells grown on nucleopore filters was monitored by metabolic labelling with a [^{35}S]methionine/[^{35}S]cysteine pulse (15 min), followed by a chase in cold medium. At

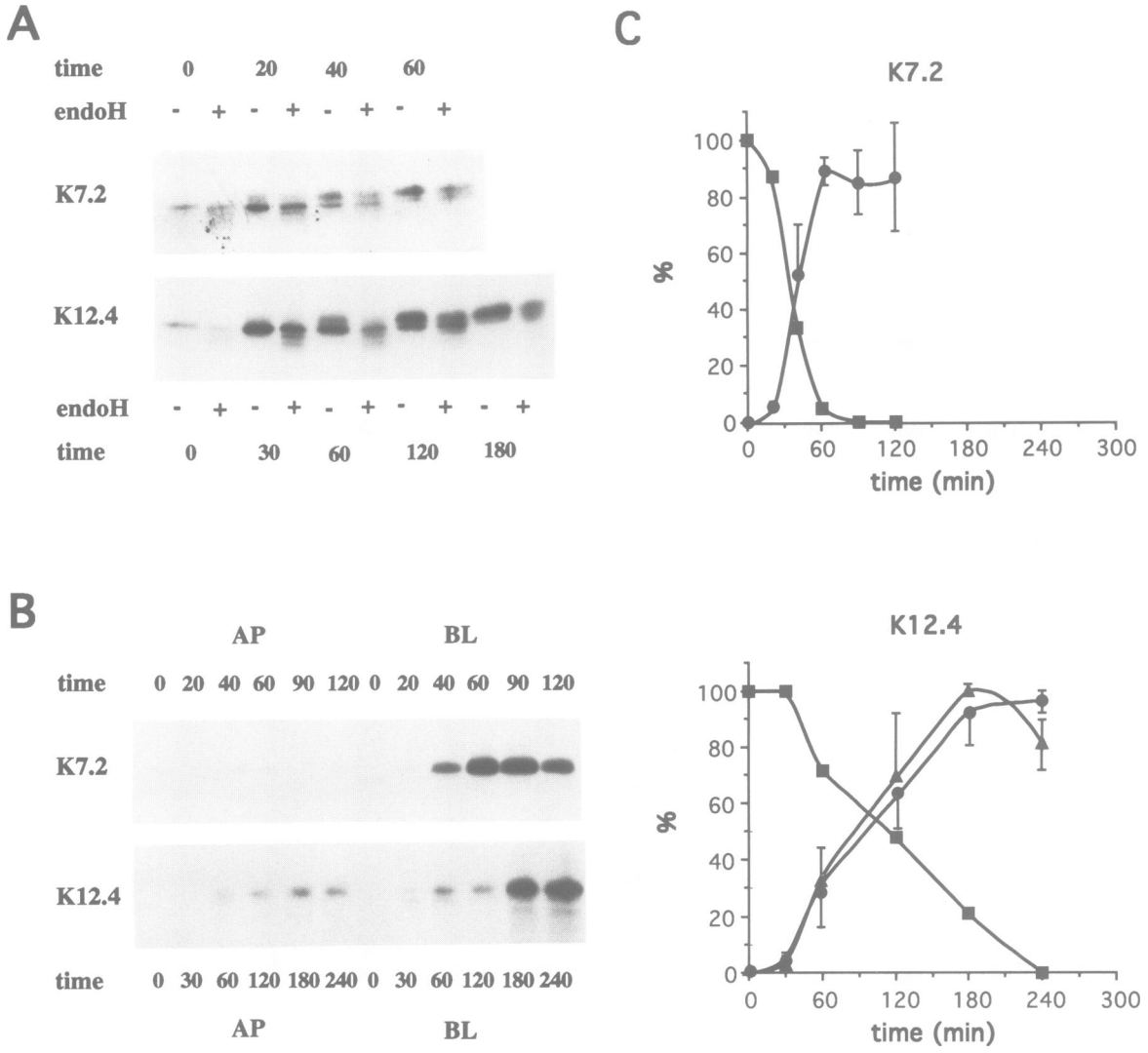

Fig. 6. Intracellular processing and appearance at the cell surface of wild-type and deletion mutant hTfR in MDCK cells. (**A**) To study hTfR glycosylation, cells were pulsed with ^{35}S-labelled methionine and cysteine for 15 min and chased for various lengths of time (indicated in min). hTfR was immunoprecipitated with B3/25 antibody, digested or not with endoglycosidase H, resolved in a 6–15% SDS–polyacrylamide gel and revealed by fluorography. (**B**) To measure the migration of hTfR to the cell surface, cells were pulsed for 15 min with biosynthetic ^{35}S-label and chased for indicated lengths of time. The apical (AP) or basolateral (BL) surfaces were then biotinylated on ice, cells lysed and hTfR immunoprecipitated. Precipitates were dissociated by boiling in SDS and biotinylated surface receptors isolated by adsorption to streptavidin–agarose. Samples were analysed by SDS–PAGE and fluorography. (**C**) Fluorograms as shown above were scanned. The kinetics of receptor maturation is represented by the disappearance of the precursor form (■), calculated as the percentage of total receptor. Values represent the average of two experiments. The kinetics of appearance at the basolateral (●) or apical (▲) surfaces were normalized to the amount at the time of maximal expression at either membrane. At the plateau, the basolateral and the apical contents of hTfR in K12.4 cells correspond to 80 and 20%, respectively, of the total surface expression. Values represent the average of three experiments.

different times of chase, chosen according to the maturation kinetics, cells were labelled either on their apical or basolateral side with sulfosuccinimidyl 2-(biotinamido) ethyl-1,3-dithiopropionate (NHS-SS-Biotin). hTfR was immunoprecipitated with B3/25 antibody, released from the beads by boiling in SDS and reprecipitated with streptavidin–agarose beads. Wild-type hTfR reached the basolateral cell surface with a half-time of 40 min (Figure 6B and C), and no expression of this molecule could be detected at the apical membrane. This experiment indicated that wild-type hTfR is delivered directly to the basolateral side in MDCK cells. The time between the disappearance of the glycosylation precursor and surface delivery of the mature receptor did not exceed 5 min (Figure 6C). The deletion mutant hTfR appeared with the same kinetics both on the apical and basolateral surfaces (Figure 6B and C) in relative amounts similar to the steady state distribution of the receptor: 80% of hTfR was expressed at the basolateral membrane and 20% at the apical surface (Figure 3). This result clearly demonstrates a direct vectorial delivery of the truncated receptor to both membrane domains. As with the wild-type receptor, a short interval was observed between the appearance of mature receptor and its surface expression, indicating that the cytoplasmic deletion did not affect the kinetics of transport from the TGN to the cell membrane.

Discussion

In the present study we addressed the question of whether the endocytic signal within the TfR, or phosphorylation of

its cytoplasmic domain, play a role in targeting newly synthesized TfR to a specific plasma membrane domain. We transfected plasmids encoding either wild-type, cytoplasmic deletion or phosphorylation site mutant hTfR into MDCK cells and isolated high expressing, stable cell lines. Wild-type hTfR was capable of binding and internalizing Tf and, like endogenous canine TfR (Fuller and Simons, 1986), was expressed almost exclusively at the basolateral membrane. Newly synthesized receptors were delivered directly from the TGN to the basolateral surface where accurate recycling maintained their polarity. Phosphorylation of hTfR is not involved in its localization to the basolateral domain since the cell surface distribution was not affected by mutation of the unique phosphorylation site Ser24 (Davis *et al.*, 1986; Rothenberger *et al.*, 1987). This result is consistent with data on polymeric immunoglobulin receptor that have shown no role of serine phosphorylation in correct basolateral delivery (Casanova *et al.*, 1990). The deletion mutant hTfR lacking cytoplasmic residues 6–41 was still mainly (80%) present at the basolateral domain, but showed an increased (20%) apical localization. Although the expression of the deletion mutant hTfR was not strictly basolateral, it can be considered, based on other examples like Fc receptor type II-B2 (Hunziker *et al.*, 1991), as clearly a polarized receptor in epithelial cells. Such a distribution was observed regardless of the level of deletion mutant hTfR expression. This excludes overexpression as a possible cause of apical missorting and argues against a role of endogenous canine receptor in targeting exogenous hTfR basolaterally. The latter conclusion is also supported by the absence of TfR heterodimers in immunoprecipitates. The polarity of MDCK cell lines was checked using the major apical sialoglycoprotein Gp114 as a control (Le Bivic *et al.*, 1990b) and was found not to be altered by the transfection. As for the wild-type hTfR, deletion mutant hTfR was vectorially delivered from the TGN to the cell surface where it was able to bind Tf but not internalize it. Although the maturation of truncated hTfR was delayed compared with the wild-type molecule, the cytoplasmic deletion did not affect the kinetics of transport from the TGN to the plasma membrane.

The cytoplasmic deletion of residues 6–41 inhibits internalization of hTfR in transfected MDCK cells, as shown previously in L cells (Rothenberger *et al.*, 1987), chicken embryonic fibroblast (Jing *et al.*, 1990) and CHO cells (Gironès *et al.*, 1991). The sequence YTRF contained in this region has been identified as a consensus signal for endocytosis via coated pits (Collawn *et al.*, 1990; Jing *et al.*, 1990). This motif has a high propensity to form tight turns in proteins and could therefore constitute a tridimensional structure interacting with coat proteins (Collawn *et al.*, 1990). While deletion of residues 6–41 of hTfR also dramatically affects its endocytosis in MDCK cells, it only partially alters its basolateral localization. In the case of Fc receptor type II (Hunziker *et al.*, 1991), influenza hemagglutinin (Brewer and Roth, 1991), lysosomal protein lgp 120 (Hunziker *et al.*, 1991) and nerve growth factor receptor (Le Bivic *et al.*, 1991), basolateral transport and internalization involve the same or overlapping cytoplasmic domain determinants. In contrast, mutations within endocytic motifs of LDL receptor (Hunziker *et al.*, 1991; Yokode *et al.*, 1992) or polymeric immunoglobulin receptor (Casanova *et al.*, 1991) did not affect their basolateral targeting. It is noteworthy that no tyrosine is required for the main basolateral location of hTfR since the cytoplasmic domain of the deletion mutant is devoid of this amino acid. In this respect, hTfR is comparable with polymeric immunoglobulin receptor for which neither tyrosine 668 nor 734 are necessary for its basolateral targeting (Okamoto *et al.*, 1992). In contrast both tyrosines 824 and 826 of the LDL receptor belong to a segment of the cytoplasmic domain involved in the basolateral expression (Yokode *et al.*, 1992). Recent studies suggest that cytoplasmic amino acid stretches located at positions 816–828 and 655–668 in LDL and polymeric immunoglobulin receptors, respectively, confer their delivery to the basolateral membrane (Casanova *et al.*, 1991; Hunziker *et al.*, 1991; Yokode *et al.*, 1992). A consensus sequence has been proposed from these two segments (RNxDxxS/TxxS) which may recognize a traffic-directing protein in polarized epithelial cells. Since 80% of truncated hTfR was still expressed at the basolateral membrane, at least part of the basolateral targeting information must be encoded by a segment outside the endocytic motif. In support of this, it has been reported that tailless receptors that have lost entirely their basolateral localization signal are usually expressed at more than 75% in the apical domain (Mostov *et al.*, 1986; Hunziker *et al.*, 1991). Our conclusion suggests that hTfR behaves similarly to LDL receptor and polymeric immunoglobulin receptor. Interestingly the sequence NADNNT/MKAN/S located at position 47–55 in the cytoplasmic domain of human and mouse TfR highly resembles the putative consensus basolateral motif. A sequence with less homology to the consensus signal is also found at positions 27–34 (RQVDGDNS) in human and mouse TfR and its deletion together with the endocytic motif leads to missorting of 20% of the truncated hTfR to the apical membrane. Two hypotheses could explain the cell surface distribution of the deletion mutant of hTfR: (i) the conformation of a unique basolateral sorting motif outside or overlapping residues 6–41 is affected by the deletion, and correct targeting of the truncated hTfR is therefore less efficient or (ii) a second basolateral targeting information is located within amino acids 6–41, and both signals cooperate to confer a strictly basolateral expression of hTfR. The existence of a putative basolateral consensus motif, shared with LDL receptor and polymeric immunoglobulin receptor, should be investigated in the future with additional mutants. A correlation with these other two receptors for which endocytic and basolateral targeting sequences are different may be relevant to studies on the molecular mechanisms of basolateral sorting in epithelial cells.

Materials and methods

Reagents

Protein A–Sepharose was purchased from Pharmacia (Uppsala, Sweden), sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin), sulfosuccinimidyl 2-(biotinamido) ethyl-1,3-dithiopropionate (NHS-SS-biotin) and streptavidin–agarose beads were from Pierce (Rockford, IL).

Cell culture

The high resistance MDCK strain I cell line (Richardson *et al.*, 1981; Fuller *et al.*, 1984) was grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum (FCS), penicillin (50 U/ml) and streptomycin (50 μg/ml). When grown on filters, 2×10^6 cells were seeded on Transwell chambers (24.5 mm diameter) from Costar (Cambridge, MA) and cultured for 5 days with changes of medium every other day.

Construction of hTfR cDNA expression vectors

The hTfR expression vector was derived from the full-length cDNA clone pcD-TR1 (Kühn *et al.*, 1984) under transcriptional control of the genomic hTfR promoter, but lacking the regulatory 3′ untranslated region (construct 9 in Owen and Kühn, 1987). Mutations in the wild-type sequence, made by oligonucleotide-directed mutagenesis according to the methods of Zoller and Smith (1983), have been described previously (Rothenberger *et al.*, 1987). They correspond to a substitution of the unique hTfR phosphorylation site, serine 24, to alanine, and a deletion of 36 amino acids (residues 6–41) in the hTfR cytoplasmic domain.

Transfection and clonal selection

Plasmids containing wild-type or mutant hTfR cDNA were transfected into MDCK cells using the calcium phosphate precipitation method (Graham and van der Eb, 1973). Each plasmid (2 μg) was cotransfected with 200 ng of a plasmid containing the hygromycin B phosphotransferase gene (Blochlinger and Diggelmann, 1984) and 10 μg of genomic carrier DNA. Cells were incubated with the precipitate for 6 h at 37°C in the presence of 200 μM chloroquine, treated for 2 min with DME containing 15% glycerol, then thoroughly washed and cultured in DME. Cells were replated 48 h later in medium with 150 μg/ml hygromycin B. After 10–15 days, resistant cells were labelled with the monoclonal antibody 5E9 against hTfR (Haynes *et al.*, 1981) and fluoresceinated goat anti-mouse IgG (Cappel, West Chester, PA). MDCK cells with high levels of hTfR were isolated by fluorescence-activated cell sorting (Kühn *et al.*, 1983) and single-cell clones obtained by limiting dilution.

Biotinylation

Biotinylation of cell monolayers on Transwells was carried out with NHS-LC-Biotin (2.5 μg/ml PBS) or NHS-SS-Biotin (2.5 μg/ml PBS) for twice 20 min at 4°C with 0.65 ml in the apical chamber or 1.35 ml in the basolateral chamber. Free biotin was blocked with 50 mM NH_4Cl in PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (Sargiacomo *et al.*, 1989).

Biosynthetic labelling

Cells grown on filters were incubated for 30 min in DME without methionine/cysteine and pulsed for 15 min in the same medium containing [^{35}S]methionine/[^{35}S]cysteine at 1 mCi/ml (tran^{35}S-label; ICN Biomedicals, Bucks, UK) and 0.5 mCi/ml [^{35}S]cysteine (NEN). Cells were washed once with DME, chased in DME containing 2× methionine/cysteine and stored at 4°C in $NaHCO_3$-free DME, 20 mM HEPES and 0.2% BSA before biotinylation. For long-term labelling, cells grown on filters were incubated overnight in DME containing 0.1× methionine/cysteine, 1% FCS supplemented with [^{35}S]methionine/[^{35}S]cysteine at 0.5 mCi/ml in the basolateral chamber.

Immunoprecipitation

After biotinylation, filters were excised and cells solubilized in 1 ml of lysis buffer (150 mM NaCl, 20 mM Tris–HCl pH 8.0, 5 mM EDTA, 1% Triton X-100, 0.2% BSA and protease inhibitors) for 1 h at 4°C. Extracts were precleared by addition of 50 μl of a *Staphylococcus aureus* slurry (Pansorbin; Calbiochem-Behring, San Diego, CA) for 30 min and centrifuged at 15 000 *g* for 15 min. Supernatants were incubated overnight with 10 mg protein A–Sepharose precoated with rabbit anti-mouse IgG (Biosys, Compiegne, France) and monoclonal antibody B3/25 (Trowbridge and Omary, 1981) against hTfR (Boehringer, Mannheim, Germany). After incubation the beads were washed. In order to recover the immunoprecipitated biotinylated antigens, beads were boiled for 5 min with 10 μl 10% SDS, diluted with lysis buffer (600 μl/tube) and centrifuged for 1 min at 15 000 *g*. Supernatants were incubated 6 h with 50 μl streptavidin–agarose beads. Finally, the beads were washed and boiled in sample buffer and analysed by SDS–PAGE on 6–15% gradient gels. Gels were treated with sodium salicylate for fluorography and dried. Alternatively, immunoprecipitated antigens from biotinylated cells were directly analysed by SDS–PAGE and detected with [^{125}I]streptavidin on nitrocellulose blots. In controls, the predominantly apical MDCK antigen gp114 was precipitated with polyclonal rabbit antibodies (Le Bivic *et al.*, 1990b).

Endoglycosidase H digestion

After immunoprecipitation, the protein A–Sepharose beads were washed, resuspended in 50 mM Tris–HCl pH 6.8, 1% SDS, 5 mM DTT and heated for 5 min at 95°C. The supernatant was recovered and supplemented with 0.15 M sodium citrate pH 5.5, protease inhibitors and 5 mU endoglycosidase H (Sigma, St Louis, MO). Samples were incubated at 37°C for 3–6 h and analysed by SDS–PAGE.

Immunostaining of hTfR and endocytosis of Tf

For immunofluorescence, MDCK cells were grown on transparent filters (Becton Dickinson, Meylan, France), fixed for 30 min in 2% formaldehyde and permeabilized with 0.075% saponin. Mouse anti-hTfR antibody B3/25 (commercial stock diluted 1/200) and rhodamine-conjugated goat anti-mouse antibodies (Immunotech, Marseille, France), diluted 1/100 were applied for 1 h each in the presence of saponin.

To visualize Tf endocytosis, MDCK cells were grown on cover-glasses for 5 days, incubated at 4°C with 60 nM FITC-Tf (generous gift from Dr Jean Salamero) for 30 min, washed and then incubated at 37°C for 10 min. Cells were fixed for 15 min in 2% formaldehyde.

Filters or cover-glasses were mounted in Moviol (Hoechst, Frankfurt, Germany) and viewed with a confocal laser scanning microscope (Wild–Leitz CLSM). A focal series of eight horizontal sections vertically spaced by 1 μm was collected from each specimen.

Immunogold labelling of hTfR

Cells were grown on nucleopore filters. Apical compartments of the wells were preincubated for 1 h at 37°C in serum-free DME containing 0.1% ovalbumin, washed and incubated for 30 min at 4°C in 1 ml ice-cold DME containing 0.1% ovalbumin and 2 μg of monoclonal antibody B3/25 against hTfR. Cells were then washed twice with ice-cold DME and incubated for a further 30 min at 4°C in 1 ml DME containing 0.1% ovalbumin and 20 μl of a suspension of 5 nm gold particles coated with protein A (Sigma). After six washes with ice-cold DME and fixation with 2.5% glutaraldehyde for 30 min at room temperature, cells were processed for electron microscopy as previously described (Iacopetta *et al.*, 1983).

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