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A di-leucine motif mediates endocytosis and basolateral sorting of macrophage IgG Fc receptors in MDCK cells

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An important function of the low affinity IgG Fc receptor FcRII-B2 (FcR) on macrophages is the internalization of soluble antigen–antibody complexes for lysosomal degradation. Most endocytic receptors possess tyrosine-containing cytoplasmic determinants required for endocytosis. In many proteins, signals which overlap with the endocytosis determinant and share the same critical tyrosine residue also mediate basolateral sorting in the *trans*-Golgi network of epithelial cells. Despite the presence of two tyrosine residues in the FcR cytosolic domain, neither one is absolutely required for coated pit localization or basolateral targeting. Nevertheless, a short domain of 13 residues containing one of the non-critical tyrosine residues mediates endocytosis and basolateral delivery. Alanine scan mutagenesis of this region now revealed a critical role of a leucine-leucine motif in both events. These findings suggest that endocytosis and basolateral sorting can be mediated by both tyrosine- and di-leucine-based signals and confirm the close relationship between the two determinants already observed for 'classical' tyrosine-dependent motifs.

Key words: clathrin-coated pit/epithelial cell polarity/Golgi complex/lysosomal transport/MDCK cells

Introduction

Cell type specific alternative splicing generates two different FcRII isoforms in macrophages and B cells, FcRII-B2 and FcRII-B1, respectively, reflecting the different functions of these receptors on the two cell types (Hibbs *et al.*, 1986; Lewis *et al.*, 1986; Ravetch *et al.*, 1986). On macrophages, FcRII mediate the internalization of soluble IgG complexes (Mellman and Plutner, 1984; Mellman *et al.*, 1984; Ukkonen *et al.*, 1986), the phagocytosis of IgG-coated particles (Mellman *et al.*, 1983) and the triggering of the synthesis and release of inflammatory mediators and cytotoxic agents (Nathan and Cohn, 1980; Unkeless *et al.*, 1981). On B cells, the function of FcR is less well characterized, but they seem to be involved in controlling B cell activation (Phillips and Parker, 1984; Cambier *et al.*, 1987; Klaus *et al.*, 1987; Hunziker *et al.*, 1990). FcRII-B1 and FcRII-B2 are identical except for an in-frame insertion of 47 amino acids in the cytoplasmic domain of the B cell isoform (Qiu *et al.*, 1990). Contrary to the FcRII-B2 on macrophages, FcRII-B1 on B cells is unable to mediate endocytosis, possibly due to the tethering of receptors to the actin cytoskeleton via the cytoplasmic tail insertion (Amigorena *et al.*, 1992; Miettinen *et al.*, 1989, 1992).

Most endocytic plasma membrane proteins and receptors have been found to accumulate in coated pits due to the presence of a tyrosine-containing determinant in their cytoplasmic domain (Davis *et al.*, 1987; Rothenberger *et al.*, 1987; Lazarovitis and Roth, 1988; Lobel *et al.*, 1989; Chen *et al.*, 1990; Collawn *et al.*, 1990; Jing *et al.*, 1990; McGraw and Maxfield, 1990; Williams and Fukuda, 1990; Canfield *et al.*, 1991; Fuhrer *et al.*, 1991; Girones *et al.*, 1991; Harter and Mellman, 1992; Lehmann *et al.*, 1992; Okamoto *et al.*, 1992). Computer modeling and two-dimensional nuclear magnetic resonance spectroscopy (NMR) analysis of peptides encoding the endocytosis signal have suggested that the critical tyrosine residue forms part of a tight turn structure (Collawn *et al.*, 1990; Bansal and Gierasch, 1991; Eberle *et al.*, 1991). FcRII-B2 possesses two tyrosine residues at positions 26 and 43 in its cytoplasmic domain (see Figure 1), but neither of these is required for coated pit localization and their substitution only slightly affects endocytosis (Miettinen *et al.*, 1992). Analysis of two cytoplasmic tail deletion mutants retaining 31 (CT31) or 18 (CT18) membrane proximal residues in the 47 amino acid long tail has suggested that the endocytosis signal is contained within a 13 residue long region between T18 and H31 (see Figure 1) (Hunziker *et al.*, 1991; Miettinen *et al.*, 1992). Interestingly, this is the only region within the cytoplasmic tail with high homology to the human counterpart (Stengelin *et al.*, 1988; Brooks *et al.*, 1989; Stuart *et al.*, 1987, 1989), consistent with an important functional role.

The same region between T18 and H31 in the FcRII-B2 tail is also of interest with respect to the polarized basolateral delivery of the receptor in MDCK cells (Hunziker and Mellman, 1989; Hunziker *et al.*, 1991). In a number of proteins, basolateral sorting and endocytosis signals are collinear and share a common critical tyrosine residue (Brewer and Roth, 1991; Hunziker *et al.*, 1991; LeBivic *et al.*, 1991; Matter *et al.*, 1992; Geffen *et al.*, 1993). At least in some proteins, however, the two overlapping signals are not identical (Hunziker *et al.*, 1991; Matter *et al.*, 1992; Prill *et al.*, 1993). In the case of FcRII-B2, the information for coated pit localization and basolateral sorting is collinear: the endocytosis competent CT31 deletion mutant is delivered basolaterally like the wild type receptor, while the non-endocytic CT18 is found apically (Hunziker *et al.*, 1991). As for the endocytosis, Y26 is not required for basolateral targeting (Hunziker *et al.*, 1991).

The goal of the present work was to analyze in more detail the structural requirements for the overlapping endocytosis and basolateral sorting signals in FcRII-B2. Using alanine scan mutagenesis, we identified a leucine-

FcRII-B1 insertion (47 amino acids)

wild type	KKKQVPDNPPDLEEAAKTEAENTITYSLLKHPEALDEETEHDYQNHI
CT31	KKKQVPDNPPDLEEAAKTEAENTITYSLLKH
CT18	KKKQVPDNPPDLEEAAKT
H31A	KKKQVPDNPPDLEEAAKTEAENTITYSLLK**A**
K30A	KKKQVPDNPPDLEEAAKTEAENTITYSLL**A**H
L29A	KKKQVPDNPPDLEEAAKTEAENTITYS**A**AKH
L28A	KKKQVPDNPPDLEEAAKTEAENTITY**A**LKH
L28A/L29A	KKKQVPDNPPDLEEAAKTEAENTITY**AA**KH
Y26A	KKKQVPDNPPDLEEAAKTEAENTIT**A**SLLKH
T25A/Y26A/S27A	KKKQVPDNPPDLEEAAKTEAENTI**AAA**LLKH
I24A	KKKQVPDNPPDLEEAAKTEAENT**A**TYSLLKH
T23A	KKKQVPDNPPDLEEAAKTEAEN**A**ITYSLLKH
N22R	KKKQVPDNPPDLEEAAKTEAE**A**TITYSLLKH
E21A	KKKQVPDNPPDLEEAAKTEA**A**NTITYSLLKH

Fig. 1. Amino acid sequences of the cytoplasmic domains of wild type and mutant Fc receptors. The sequences are shown in the single-letter code and the alanine residues substituting for amino acids in the wild-type sequence are in bold. Residues are numbered from left to right, with position 1 corresponding to the presumed first amino acid of the cytoplasmic domain.

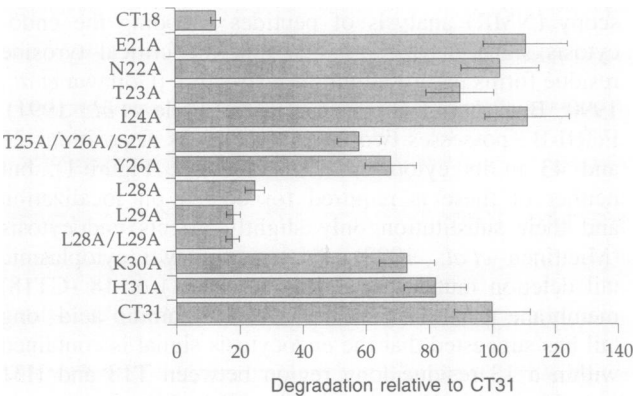


Fig. 2. IgG complex degradation by MDCK cells expressing mutant FcR. Radioiodinated IgG complexes were prebound on ice to MDCK cells expressing mutant FcR. After washing unbound ligand, cells were transferred to 37°C for 60 min to allow endocytosis and degradation to occur. The medium was collected, TCA precipitated, and the radioactivity of the TCA supernatant, the pellet and the cells was determined. IgG complex degradation (TCA-soluble radioactivity in the media) was normalized to total initially bound ligand (6–9 × 10^4 c.p.m.). For CT31, this value was in the 25% range and was arbitrarily set to 100% and the relative degradation activity of the FcR mutants as compared with that of CT31 was plotted. Determinations were performed in duplicate.

leucine motif required for both sorting events. While a di-leucine sequence has previously been implied in mediating endocytosis and lysosomal transport (Johnson and Kornfeld, 1992; Letourneur and Klausner, 1992), this motif has not been shown to be utilized by classical endocytic receptors. Furthermore, the role of the di-leucine motif in polarized sorting is novel and our finding enforces the close relationship between coated pit localization and basolateral targeting already found for 'classical' tyrosine-dependent signals. In addition, our results show that tyrosine- and di-leucine-based signals are capable of segregating proteins not only into the endocytic and lysosomal, but also into the basolateral pathway of polarized cells.

Results

Generation of FcRII-B2 tail mutants and expression in MDCK cells

Based on the analysis of two truncation mutants carrying 18 (CT18) or 31 (CT31) membrane-proximal residues, the information required for endocytosis and basolateral sorting in MDCK cells has been localized to the region between T18 and H31 in the cytoplasmic domain of FcR (see Figure 1). While CT31 was delivered to the basolateral surface and mediated endocytosis as efficiently as the wild type receptor, the CT18 truncation yielded an endocytosis-deficient receptor that was delivered to the apical domain (Hunziker *et al.*, 1991; Miettinen *et al.*, 1992). In order to characterize further the structural features required for internalization and basolateral sorting, we analyzed the critical region in CT31 by alanine scan mutagenesis.

As outlined in Figure 1, single or multiple amino acids between T18 and H31 were replaced by alanine residues in CT31 and the mutant receptors were expressed in MDCK cells. Cells expressing FcR were enriched by fluorescence activated cell sorting using a fluorescein-labeled anti-receptor monoclonal antibody, 2.4G2 (Unkeless, 1979), yielding populations with 70–100% of the cells expressing the receptor. The data for the sorted populations are shown, although similar results were also obtained for non-enriched populations containing only 20–30% FcR-positive cells.

Lysosomal degradation of IgG complexes requires two adjacent leucine residues in the cytoplasmic domain of FcR

To obtain a first indication as to the importance of the various amino acid substitutions in endocytosis, we analyzed the ability of MDCK cells expressing mutant FcR to internalize and degrade IgG complexes (Hunziker and Mellman, 1989). Radiolabelled IgG complexes consisting of rabbit anti-DNP and [^{125}I]DNP-BSA were allowed to bind to cells on ice. After washing unbound ligand, cells were transferred to 37°C to allow ligand internalization and degradation to occur. Degradation was measured as the release of TCA-soluble radioactivity into the medium after 60 min. As previously shown and similar to cells expressing wild-type FcR, ~25% of surface-bound IgG complexes were degraded by cells transfected with CT31, while <10% degradation occurred in cells carrying CT18 (Hunziker *et al.*, 1991). In Figure 2, the degradation obtained by the cells expressing the various substitution mutants is shown normalized with respect to the degradation of CT31-expressing cells. Interestingly, all mutants except those carrying substitutions of L28 and/or L29 were capable of mediating degradation with efficiencies ranging between 60% and 115% of the CT31-expressing control cells. Substitution of Y26 alone or together with the surrounding residues (T25 and S27) moderately inhibited degradation by ~40%, consistent with results obtained for a Y26 mutant in the context of the full-length receptor (Miettinen *et al.*, 1992). In contrast, degradation by receptors carrying substitutions affecting L28 and L29, either alone or together, was reduced by >80%, to levels comparable to those obtained for CT18.

Thus, these results suggest an important role of L28 and L29 in endocytosis and/or lysosomal delivery of FcR.

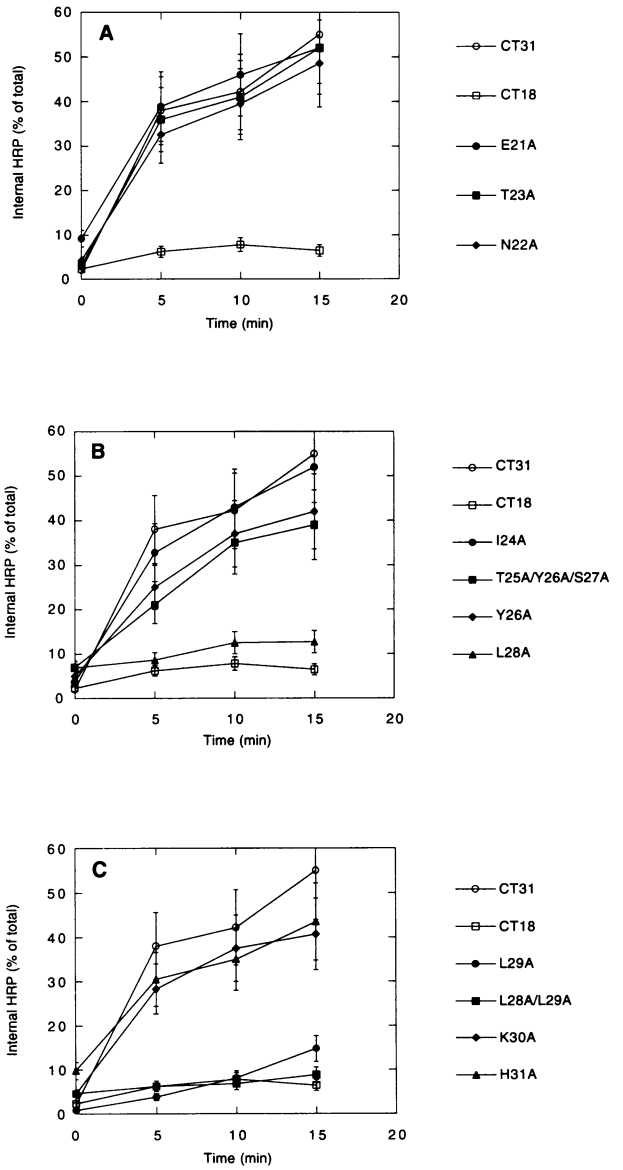


Fig. 3. Kinetics of IgG complex internalization by MDCK cells expressing mutant FcR. Rabbit anti-HRP–HRP complexes were bound on ice to MDCK cells expressing mutant FcR. After washing, cells were transferred to 37°C to allow internalization of immune complexes. Cells were returned on ice and endocytosis was determined by measuring the amount of total versus surface HRP activity in permeabilized or intact cells, respectively. Internalized anti-HRP–HRP was calculated by subtracting the amount of surface HRP activity from that of total HRP activity. Determinations were performed in triplicate.

The di-leucine motif is required for rapid and efficient endocytosis

We next analyzed the effect of the amino acid substitutions on the early kinetics of internalization. IgG complexes, formed using horseradish peroxidase (HRP) and anti-HRP antibody, were bound to cells on ice. After removing unbound ligand, cells were warmed to 37°C to allow internalization for various periods of time. Cells were cooled and surface-bound and total HRP was determined by measuring the HRP activity in the absence or presence of detergent, respectively. The amount of internalized ligand was calculated from the difference between these two values (Drake *et al.*, 1989; Miettinen *et al.*, 1992).

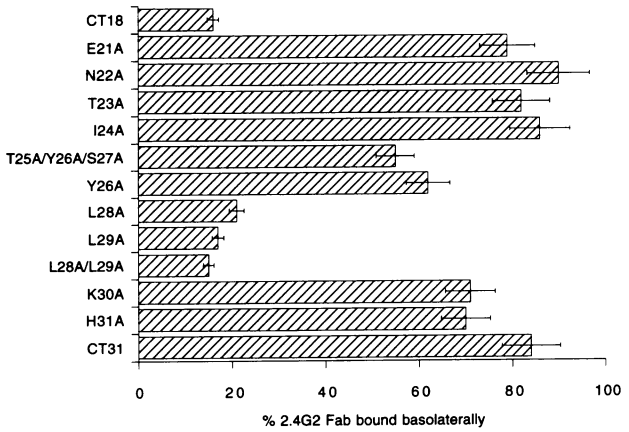


Fig. 4. Polarized cell surface distribution of mutant FcR in polarized MDCK cells. Cells grown on Transwell units were allowed to bind radioiodinated Fab fragments of the monoclonal anti-FcR antibody 2.4G2 either from the apical or the basolateral compartment on ice. Unbound antibody was removed by washing, filters were cut out and bound radioactivity determined. Values for non-specific binding, determined in the presence of a 100-fold excess of unlabeled 2.4G2 IgG, were subtracted and represented 10–15% of the total binding. Total binding ranged from 2–6 × 10^4 c.p.m. Experiments were carried out in duplicate.

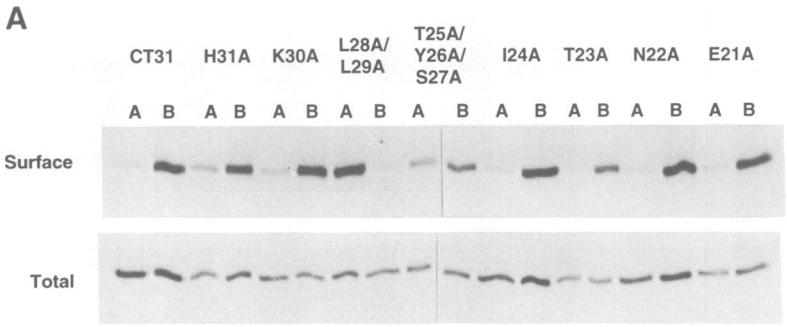
As shown in Figure 3A, CT31 rapidly and efficiently internalized ligand, with up to 50% of the prebound IgG complexes being intracellular after 5 min. Mutations affecting Y26 either alone or in conjunction with T25 and S27 (Figure 3B), displayed a moderate but reproducible reduction of the initial kinetics and the final extent of endocytosis, consistent with previous observations (Miettinen *et al.*, 1992). Similarly, substitution of K30 and H31 resulted in a slight but consistent inhibitory effect (Figure 3C). However, only substitutions affecting one or both leucine residues at positions 28 and 29 displayed a dramatic and almost complete inhibition of endocytosis, with both the initial rate as well as the final extent of ligand internalization being reduced to levels comparable to those of CT18 (Figure 3B and C). Since no other mutation affected endocytosis to the extent found for the L28 or L29 substitutions, the latter two residues must predominantly contribute to the endocytic activity of CT31.

These results are therefore consistent with the degradation experiments and suggest that the two leucine residues play a crucial role in FcR endocytosis.

The di-leucine motif specifies basolateral localization of FcR in MDCK cells

Since the region between T18 and H31 in the cytoplasmic tail of FcR also encodes the information required for basolateral delivery in MDCK cells (Hunziker *et al.*, 1991), we next analyzed the steady-state distribution of FcR on the apical and basolateral surfaces of polarized MDCK cells expressing the different mutants. Cells were grown on Transwell units to obtain tight monolayers of polarized cells and radiolabeled Fab fragments of the anti-FcR antibody 2.4G2 were allowed to bind on ice from the apical or basolateral compartment (Hunziker and Mellman, 1989). After removing non-bound Fab fragments, radioactivity bound from the two compartments to the cell monolayer was determined.

Confirming previous observations (Hunziker *et al.*,



B

Mutant	Basolateral insertion (%)
CT31	89 ± 9
H31A	65 ± 11
K30A	79 ± 9
L28A/L29A	8 ± 7
T25A/Y26A/S27A	62 ± 13
I24A	90 ± 6
T23A	92 ± 6
N22A	89 ± 7
E21A	90 ± 5

Fig. 5. (**A**) Polarized cell surface insertion of newly synthesized mutant FcR. MDCK cells expressing mutant FcR and grown on Transwell units and pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine for 15 min and then chased for 30 min. Biotinylated Fab fragments of the monoclonal anti-FcR antibody 2.4G2 were included during the chase either in the apical or basolateral compartment. After the chase, cells were washed on ice, lysed and labeled FcR that had appeared on the cell surface (and thus bound biotinylated Fab fragments) were precipitated with streptavidin–agarose (surface). Total FcR was precipitated from an aliquot of the lysate with a polyclonal anti-FcR serum (total). Samples were treated with endoglycosidase F and analyzed by SDS–PAGE and autoradiography. (**B**) Quantification. The signals on autoradiographs of two independent experiments were quantified by densitometry and the amount of surface receptor was normalized to total labeled FcR. The fraction of each mutant inserted into the basolateral domain is shown.

1991), ~80% of Fab-binding occurred from the basolateral surface in CT31-expressing cells, while cells carrying CT18 predominantly bound Fab from the apical side (Figure 4). Mutants with substitutions affecting T25/Y26/S27 or Y26, as well as K30 or H31, displayed a less polarized basolateral localization, but nevertheless, 60–75% of these mutants were still found on the basolateral surface. Substitutions targeting L28 and/or L29, however, lead to a reversal of polarity, with >80% of the receptors now being on the apical surface. Importantly, no other substitution resulted in a similar reversal of polarity and the almost exclusive apical polarization of the L28 and L29 substitutions was similar to that of CT18, consistent with a crucial role for the leucine-leucine sequence in determining the basolateral localization of CT31.

The Fab-binding results were confirmed by experiments where the apical or basolateral domain of metabolically labeled cells was surface biotinylated and surface FcR were detected by immunoprecipitation followed by adsorption to streptavidin–agarose (not shown).

Taken together, these experiments are consistent with the di-leucine motif playing the critical role in the basolateral localization of FcR in MDCK cells.

Newly synthesized FcR mutants are vectorially inserted into the respective plasma membrane domains

The steady-state distribution of a given protein on the apical or basolateral membrane is the result of both biosynthetic delivery and transcytosis [for a review see Hunziker and Mellman (1991)]. Since FcRII-B2 is capable of mediating apical to basolateral transcytosis (Hunziker and Mellman, 1989; Hunziker *et al.*, 1990), we next analyzed whether the different polarized distributions of the CT31 mutants reflected the direct polarized delivery of newly synthesized receptors from the *trans*-Golgi network (TGN) to the respective plasma membrane surface.

For this purpose, cells grown on Transwell units were pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine and labeled proteins were chased to the cell surface for 35 min, the time taken for the first labeled FcR molecules to appear on the cell surface (Hunziker *et al.*, 1991). To detect proteins arriving at either the apical or basolateral cell surface, Fab fragments of biotinylated anti-FcR monoclonal antibody 2.4G2 were included in the apical or basolateral chamber during the chase. Cells were lysed and labeled FcR bound to biotinylated Fab fragments isolated by adsorption to immobilized streptavidin (Hunziker *et al.*, 1991).

As shown in Figure 5A and B, ~85–90% of CT31 could be detected on the basolateral surface during the 30 min chase. In contrast, the bulk of receptors carrying a substitution of the di-leucine motif were inserted into the apical domain, with only ~20% being delivered basolaterally. All other mutant receptors analyzed preferentially bound biotinylated Fab from the basolateral compartment, suggesting that they were predominantly delivered from the TGN to the basolateral domain without previous appearance on the apical surface. As found for the steady-state distribution (see Figure 4), a slightly larger fraction of newly synthesized receptors carrying a substitution of T25/Y26/S27, K30, or H31 was delivered to the apical domain (35–40%) as compared with control CT31 receptors (15–20%). These experiments therefore confirm the polarized localization of the different mutants at equilibrium (see above) and show that their distribution reflects the vectorial insertion of newly synthesized receptors into the respective domains.

In conclusion, these results show that the di-leucine motif plays a critical role in the polarized sorting of FcR from the TGN into the basolateral pathway of MDCK cells.

Discussion

The di-leucine motif as an endocytosis signal

Specific signals in the cytoplasmic domain of endocytic receptors mediate their inclusion into clathrin-coated pits [reviewed by Vaux (1992)]. The common feature of these signals is that they encode a critical tyrosine residue which forms part of a tight turn structure. One interesting exception to this rule is the macrophage FcRII-B2. Alanine scan mutagenesis of the domain encoding the endocytosis information now revealed a critical role for a leucine-leucine motif in internalization of this receptor. A similar

motif has been found to mediate endocytosis and lysosomal sorting of CD3-γ and CD3-δ chains (Letourneur and Klausner, 1992), as well as sorting of mannose 6-phosphate receptor in the Golgi (Johnson and Kornfeld, 1992). However, this is the first instance to our knowledge where a typical endocytic receptor was found to utilize this signal. As would be expected from the critical role of the di-leucine sequence in FcR endocytosis, the motif is conserved in the human homologue hFcRII-C (Stuart *et al.*, 1987, 1989; Stengelin *et al.*, 1988; Brooks *et al.*, 1989). Whether di-leucine endocytosis signals are also functional in other endocytic receptors is not known. However, since the intestinal IgG Fc receptor of newborn rats lacks cytoplasmic aromatic residues, endocytosis could be mediated by the di-leucine motif present in the tail of this receptor (Simister and Mostov, 1989).

The HA-II adaptor complex found in clathrin-coated pits on the plasma membrane has been suggested to be the cytoplasmic receptor with which tyrosine-containing internalization signals interact (Glickman *et al.*, 1989; Beltzer and Spiess, 1991; Sosa *et al.*, 1993). Since it is well established that endocytosis of FcR (and CT31) occurs via clathrin-coated pits (Miettinen *et al.*, 1992; reviewed by Mellman *et al.*, 1992), the di-leucine motif might mediate interaction with the same adaptins as the tyrosine-based signals. Even for the well characterized tyrosine-dependent determinants, it remains to be determined exactly what residues in the signal are being recognized. Although in some proteins the critical tyrosine can be replaced by other aromatic amino acids without affecting endocytosis (Davis *et al.*, 1987; McGraw and Maxfield, 1990; Canfield *et al.*, 1991), the stability of the turn is reduced (Bansal and Gierasch, 1991). Thus, it is conceivable that the tyrosine residue is more important for stabilizing the secondary structure of the signal rather than being involved in the direct interaction between receptor and adaptor complex, although direct evidence supporting this notion is not available. In addition to the tyrosine residue, bulky hydrophobic side chains such as those provided by the di-leucine motif seem to be an important feature in some 'classical' coated pit signals (reviewed by Vaux, 1992). Whether the di-leucine signal in the FcR can acquire the secondary structure of a tight turn remains to be determined, but, at least superficially, the sequence comprising Y26 and the two leucine residues resembles that of classical signals and it is conceivable that either Y26 or other residues help to stabilize a tight turn. That a tight turn structure can be obtained independently of a tyrosine residue has recently been suggested for the basolateral sorting determinant of the pIgR (Aroeti *et al.*, 1993) (see below). Although mutation of Y26 and surrounding residues in FcRII-B2 shows partial effects on endocytosis and basolateral sorting, the tyrosine residue is unable to mediate these events in the context of the leucine mutants, suggesting that Y26 does not encode a second weak signal that functions independently of the di-leucine motif. Rather, Y26 could play a less critical role as a part of the di-leucine signal, either in stabilizing a turn structure, or in modulating the affinity of the interaction with adaptor complexes. Unfortunately, it is difficult to draw conclusions with respect to the exact contribution of neighboring amino acids to the signal since alanine scan mutagenesis only examines the ability of a certain position to tolerate this particular substitution.

The di-leucine motif as a basolateral sorting signal in polarized cells

Similar to the signal-mediated clustering of endocytic receptors into clathrin-coated pits, polarized sorting of proteins to the basolateral surface of epithelial cells requires the presence of distinct cytoplasmic tail determinants (reviewed by Hunziker and Mellman, 1991; Mostov *et al.*, 1992; Mellman *et al.*, 1993). These signals appear to belong to two classes: those that are collinear with the endocytosis information (Brewer and Roth, 1991; Hunziker *et al.*, 1991; LeBivic *et al.*, 1991; Matter *et al.*, 1992; Geffen *et al.*, 1993; Thomas *et al.*, 1993), and those that reside on a separate region (Casanova *et al.*, 1991; Matter *et al.*, 1992; Okamoto *et al.*, 1992; Yakode *et al.*, 1992; Dargemont *et al.*, 1993). For both classes, tyrosine-dependent and -independent signals have been described. Where basolateral signals overlapping with the coated pit determinant have been analyzed in more detail, the two were found to be distinct (Hunziker *et al.*, 1991; Matter *et al.*, 1992; Prill *et al.*, 1993). For FcRII-B2 we have now found that the same di-leucine motif required for endocytosis also mediates basolateral delivery. Interestingly, mutation of the same residues that showed slight effects on endocytosis also partially reduced basolateral localization. However, since alanine scan mutagenesis fails to determine exactly which of the adjacent residues are essential or merely permissive, it is not possible to tell conclusively from our results whether the two signals are indeed identical.

Two other tyrosine-independent basolateral sorting signals have so far been described for the polymeric immunoglobulin receptor (pIgR) and the transferrin receptor. In contrast to the FcR, the basolateral sorting information in these two receptors is not collinear with the coated pit domain (Casanova *et al.*, 1991; Okamoto *et al.*, 1992; Dargemont *et al.*, 1993). NMR analysis of peptides encoding the basolateral sorting signal of the pIgR have recently shown that this signal might form a tight turn structure (Aroeti *et al.*, 1993). Intriguingly, a bulky hydrophobic residue (valine at position 660), is particularly important for basolateral sorting of the pIgR and also forms part of the tight turn. Therefore, one common element of the tyrosine-independent basolateral sorting signals in the pIgR and the FcR seems to be the requirement of one or more bulky hydrophobic residues, possibly located in a tight turn. In the transferrin receptor, two valine residues are found in the region implicated in sorting (Dargemont *et al.*, 1993) and it will be of interest to determine whether they play a role in basolateral delivery of this receptor. Finally, in the case of the asialoglycoprotein receptor, basolateral sorting is only partially affected by mutating the tyrosine critical for endocytosis (Geffen *et al.*, 1993) and a membrane-proximal di-leucine sequence could be responsible for the residual basolateral sorting activity of the tyrosine mutant.

Multiple clustering motifs mediate endocytic, lysosomal and basolateral sorting

At least two generic sorting motifs seem to specify the fate of integral proteins in the peripheral membrane

system. Tyrosine-dependent signals specify direct transport form the TGN to endosomes/lysosomes, endocytosis from the cell surface, and sorting in the TGN of polarized cells into basolateral carrier vesicles. Interestingly, our results now show that also di-leucine-based motifs are able to mediate segregation into precisely the same pathways as the tyrosine-containing determinants. Tyrosine-based signals most likely represent a family of related but distinct determinants (Hunziker *et al.*, 1991; Matter *et al.*, 1992; Prill *et al.*, 1993) and it remains to be shown whether this is also the case for di-leucine motifs, Although our results are consistent with the endocytosis and basolateral determinants in FcR being identical, they do not prove it. While the di-leucine signals in the CD3 γ and δ chains were able to mediate Golgi sorting and endocytosis (Letourneur and Klausner, 1992), the motif in the mannose 6-phosphate receptor was only required for Golgi sorting and did not play a role in internalization (Johnson and Kornfeld, 1992).

The apparent redundancy in sorting information for the pathways mentioned above raises a number of intriguing questions. It will be of interest to see whether the secondary structure of the two motifs is related and whether they interact with the same set of cytosolic proteins (adaptins?). If they do, they may differ in some aspect of their interaction such as affinity. If the interaction occurs with different coat proteins, the two types of signal could mediate transport to the same final destination, but via different pathways, i.e. direct delivery from the TGN to the cell surface versus indirect transport via endosomes, or direct delivery from the TGN to endosomes/lysosomes versus transport via the cell surface. As suggested by Letourneur and Klausner (1992), there might be cooperativity between the two motifs, such that the presence of one type of signal alone could mediate transport form the TGN to the cell surface, whereas both motifs could lead to direct lysosomal delivery. Finally, the significance of the two motifs in polarized sorting and whether they specify distinct basolateral pathways [i.e. one from the TGN directly to the basolateral domain, the other via endosomes; see Hopkins (1991) and Hunziker and Mellman (1991)] will need to be addressed.

Materials and methods

Construction of FcRII-B2 mutants and expression in MDCK cells

To mutagenize the cytoplasmic FcR domain, the polymerase chain reaction (PCR) was carried out using the deletion mutant CT31 in the expression vector pCB6 (Hunziker *et al.*, 1991) as template. A sense primer covering the unique *ApaI* site in FcRII-B2 was combined with different mutagenic antisense primers carrying *XbaI* sites in the non-coding region. The sequence of the primers is available upon request. PCR fragments were cut with *ApaI–XbaI* and used to replace the *ApaI–XbaI* fragment of CT31 in Bluescript. The sequence of all fragments synthesized by PCR was verified by dideoxy sequencing. MDCK cells were stably transfected as described (Hunziker and Mellman, 1989). Each transfection yielded 100–200 geneticin-resistant clones and cells expressing FcR were enriched by fluorescence activated cell sorting using FITC-labeled anti-receptor antibody 2.4G2 IgG (Unkeless, 1979). MDCK cells were cultured on plastic or on permeable polycarbonate filters (Transwell, Costar Co.) as detailed (Hunziker and Mellman, 1989).

IgG complex internalization and degradation

Internalization of rabbit anti-HRP–HRP complexes was carried out exactly as described before (Miettinen *et al.*, 1992). Briefly, complexes (20 μg/ml) were bound to cells on ice and after washing excess ligand, cells were warmed to 37°C for different periods of time. Cells were returned to ice and surface-bound and total HRP was determined by measuring the HRP activity in the absence or presence of detergent. Internalized ligand was calculated from the difference between these two values (Drake *et al.*, 1989). Degradation of rabbit anti-DNP–[^{125}I]DNP-BSA complexes was carried out exactly as previously described (Hunziker and Mellman, 1989; Hunziker *et al.*, 1991; Miettinen *et al.*, 1992). Complexes (20 μg/ml) were prebound on ice, non-bound ligand was removed by washing, and cells were allowed to internalize and degrade complexes for 60 min at 37°C. Degradation was defined as the fraction of the initially bound radioactivity that became soluble after TCA precipitation of the incubation medium.

Polarized steady-state distribution and surface delivery of mutant FcR

Binding of radioiodinated 2.4G2 Fab fragments to the basolateral or apical surface of MDCK cells grown on polycarbonate filters was carried out as outlined (Hunziker and Mellman, 1989). Non-specific binding (<10%), determined in the presence of a 100-fold excess of unlabeled 2.4G2 IgG, was subtracted from the values. To determine the polarized insertion of newly synthesized FcR into the apical or basolateral surface, cells were pulse-labeled with 1 mCi/ml radioactive methionine/cysteine (^{35}S-EXPRESS, New England Nuclear) and chased for various periods of time. Biotinylated Fab fragments of the anti-FcR antibody 2.4G2 (2 μg/ml) were included in the apical or basolateral compartments during the chase. Cells were lysed and labeled FcR that had reached the surface and thus had bound biotinylated Fab fragments were isolated using streptavidin–agarose, exactly as outlined (Hunziker *et al.*, 1991). Total labeled receptors were precipitated using an anti-FcR serum (kindly provided by Ira Mellman, Yale University).

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