Abstract. In MDCK cells, basolateral sorting of most membrane proteins has been shown to depend on distinct cytoplasmic domain determinants. These signals can be divided into those which are related to signals for localization at clathrin-coated pits and those which are unrelated. The LDL receptor bears two tyrosine-containing signals, one of each class, that can independently target receptors from the Golgi complex and from endosomes to the basolateral plasma membrane. We have now investigated the other structural features required for the activity of both determinants. We find that both depend, at least in part, on clusters of 1-3 acidic amino acids located on the COOH-terminal side of each tyrosine. While single residues adjacent to each tyrosine were also found to be critical, the two signals differed in that only the coated pit-unrelated signal could tolerate a phenylalanine in place of its tyrosine residue. We also found that the structural requirements for basolateral targeting of the "coated pit-related" signal were distinct from those required for rapid endocytosis. Apart from sharing a common tyrosine residue, no feature of the NPXY motif for coated pit localization was required for basolateral targeting. We also investigated basolateral targeting of the mouse macrophage Fc receptor (FcRII-B2) which contains a tyrosine-independent coated pit localization signal. Basolateral transport and endocytosis were found to depend on a common di-leucine-type motif. Thus, basolateral targeting determinants, like coated pit domains, can contain either tyrosine- or di-leucine-containing signals. The amino acids in the vicinity of these motifs determine whether they function as determinants for endocytosis, basolateral targeting, or both.
(Matter et al., 1992), the polymeric immunoglobulin receptor (plg-R) (Casanova et al., 1991), and the transferrin receptor (Dargement et al., 1993, unpublished). In the case of LDL-R, this second determinant is also strongly dependent on a single tyrosine residue although this signal has no activity with respect to coated pit localization (Matter et al., 1992). In contrast, the plg-R determinant appears to be only weakly tyrosine dependent (Aroeti et al., 1993). Although the structural relationships, if any, among these various determinants are not clear, their sequences have yet to be sufficiently well characterized to allow meaningful comparisons. Also unknown is whether the coated pit-related and -unrelated signals mediate basolateral transport by sorting membrane proteins into common or distinct basolateral pathways.

The human LDL-R has proven to be an excellent example since its cytoplasmic domain contains both types of basolateral targeting determinants, with the coated pit-unrelated signal being distal to the membrane-proximal coated pit-related signal (Fig. 1). Both determinants mediate basolateral sorting on the biosynthetic pathway as well as in endosomes (Matter et al., 1993). Although each depends on a critical tyrosine for activity, they can be differentiated functionally since basolateral targeting via the proximal (coated pit-related) signal is saturated by receptor overexpression; targeting via the distal (coated pit-unrelated) signal is equally efficient at both low and high expression levels (Matter et al., 1992). The proximal signal can also be distinguished from the coated pit localization signal since basolateral targeting requires amino acid residues COOH-terminal to the tyrosine that are not required for endocytosis.

We have now analyzed the structural features of the two basolateral-sorting determinants in detail. We have found that they do not only share a dependency on a tyrosine, but also require COOH-terminal acidic amino acids for activity. These features define a common motif and suggest that coated pit-related and -unrelated signals in LDL-R are fundamentally similar. Apart from the tyrosine dependence, neither signal was found to bear any structural relationship to the LDL-R's coated pit localization domain. Such a relationship was observed, however, in the case of the mouse macrophage Fc receptor whose coated pit localization domain and basolateral targeting signal were found to rely on a common di-leucine-containing motif.

Materials and Methods

Cell Culture

MDCK (strain II) cells were grown as described (Hunziker and Mellman, 1989; Matter et al., 1992). Costar Transwell polycarbonate filter units were generously provided by Hand Lane (Corning-Costar Corp., Cambridge, MA), and for experiments tissue culture-treated units with a pore size of 0.4 µm were used. Cells were plated 4-5 d before the experiments (2 x 10⁶ cells per 24-mm U and 2 x 10⁵ cells per 7.5 mm U).

Mutagenesis and Transfection

All mutants of the LDL-R, FcRII-B2, and chimeric receptors were constructed as described previously (Matter et al., 1992). The sequences of all cloned fragments synthesized by PCR were confirmed by dyeoxyn sequencing. The sequences of all primers are available on request. cDNAs encoding mutant LDL-R and chimeric receptors were cloned into the CMV-based expression plasmid pCB6. Cells were transfected and tested for expression and polarity as described previously (Hunziker and Mellman, 1989; Matter et al., 1992). For each mutant, an uncloned cell line and at least two clones were analyzed. Experiments were performed both with and without induction by sodium butyrate (0-10 mM sodium butyrate for 12-16 h).

Binding of Radioiodinated Antibodies to MDCK Cells

Binding assays were essentially performed as described previously (Matter et al., 1992). For cells expressing receptors with the extracytoplasmic domain from the LDL-R monoclonal antibody, C7 IgG (Beisiegel et al., 1981) was used while for receptors with the ectodomain from FcRII was probed with Fab fragments obtained from monoclonal antibody 2.4G2 (Unkeless, 1979). If C7 IgG was used, cells were starved in serum-free medium for 15 min before the experiment. Cells were cooled on ice and antibody was bound for 2 h from either the apical or basolateral side in PBS containing 0.5% BSA (2 µg/ml, 2-5 x 10⁶ cpm/µg). Tightness of monolayers was assessed by measuring the radioactivity in the medium of both chambers; generally, <0.5% of the added radioactivity diffused through the monolayer. Unbound antibody was removed by washing five times with PBS containing 0.2% BSA. Fc receptors were excised and cell-associated radioactivity was measured in a gamma counter. Non-specific binding was determined either by preincubation with a 100-fold excess of cold antibody. Both of these methods gave similarly low values (800-1,200 cpm) which were subtracted from the cell-associated radioactivity determined for expressing cells. All data shown represent mean values derived from at least three different experiments each performed in duplicate.

Cell Surface Biotinylation

Cell surface appearance of metabolically labeled Fc receptors was assayed by cell surface biotinylation essentially as previously described, except that the biotinylation reaction was performed using 1 mg/ml NHS-LC-biotin (Pierce Chemical Co., Rockford, IL) (Matter et al., 1992, 1993).

Endocytosis Mediated by Receptors with LDL-R Ectodomains

Endocytosis mediated by receptors with LDL-R ectodomains was measured by binding radioiodinated monoclonal antibody C7 IgG (2 µg/ml in PBS containing 0.5% BSA) on ice to MDCK cells grown in 12-well dishes to ~70% confluence. After 1 h, unbound antibody was removed by washing five times with PBS and cells were incubated for various periods of time in complete DMEM containing 10 mM Hepes, pH 7.4. After cooling on ice, antibody remained at the cell surface was removed by washing twice for 5 min with 0.2% BSA in DMEM adjusted to pH 2.8 for C7 IgG. This treatment removed 90-95% of the antibody bound on ice without warming. Internalized antibody was defined as the fraction of antibody initially bound and not removed by the low pH wash. In some experiments, the radioiodinated anti-LDL-R antibody was added to cells at 37°C. After 1 h, unbound antibody was removed and surface-bound antibody acid-elicited. Then, internalized antibody was calculated as the fraction of total bound radioactivity (acid-elicited and -resistant) which was acid-resistant.

Endocytosis Mediated by Fc Receptors

Endocytosis mediated by Fc receptors was assessed by measuring uptake of HRP-IgG complexes as described for transfected fibroblasts (Miettinen et al., 1992). Briefly, preformed HRP-IgG complexes (2 µg/ml) were bound to transfected MDCK cells (~70% confluence in 24-well dishes) on ice. After 1 h, cells were washed and incubated for different intervals of time at 37°C. Then, the cells were cooled down again and incubated with or without a 0.25% Triton X-100 for 10 min. Thereafter, HRP activity was measured using o-phenylenediamine dihydrochloride as a substrate. The samples were incubated for maximally 10 min during which time the reaction remained linear. Absorbance at 492 nm of 200-µl aliquots was measured using a Titertek Multiscan plate reader. The amount of internalization was determined by calculating the difference in absorbance measured with and without Triton X-100.

Results

Downstream Negatively Charged Amino Acids Are Important for Activity of Both Basolateral Targeting Signals in LDL Receptor

We previously found that both the proximal and distal
basolateral targeting determinants in LDL-R were inactivated by deletions of amino acids on the COOH-terminal sides of each critical tyrosine residue (Matter et al., 1992). In both cases, these deletions removed clusters of three negatively charged amino acids, suggesting that these residues may be important elements of the targeting signals. To test this possibility, we generated mutants in which the negatively charged amino acids were substituted by point mutations. Mutant receptors were stably expressed in MDCK cells and their polarity determined by binding a radiiodinated LDL-R-specific antibody ($^{125}$I-C7) to the apical or basolateral surfaces of cells grown on Costar Transwell filters (Matter et al., 1992).

A mutant receptor whose proximal signal was inactivated by a point mutation of the tyrosine at position 18 (Fig. 1 A: Y-A18) was, as expected, still expressed basolaterally due to the presence of the distal determinant (Fig. 2 A). If in addition to the Y-A18 mutation, the cluster of three acidic amino acids was deleted by removing the COOH-terminal 8 residues (CT42Y-A18), the mutant receptor was expressed >90% apically (Matter et al., 1992). A less complete but nevertheless marked decrease in basolateral expression also occurred if the acidic residues at positions 46-48 (EDD) were replaced by alanines (YEDD-A18A46A47A48; ~70% apical). The reduced basolateral expression reflected a decrease in sorting efficiency of newly synthesized receptors in the TGN as determined by monitoring the appearance of newly synthesized receptors on the plasma membrane by surface biotinylation (Matter et al., 1992) (Fig. 2 C). Thus, these negatively charged amino acids appear to be important for the activity of the distal basolateral-targeting determinant of the LDL-R.

We next determined if the three acidic residues (EDE) downstream from the tyrosine at position 18 were similarly required for the activity of the proximal basolateral signal. As shown in Fig. 2 B, a deletion mutant (CT27) that removed the entire distal signal but retained the three acidic residues close to tyrosine 18 was expressed largely on the basolateral surface. Since the proximal signal was less efficient than the distal signal at high receptor expression levels, the distribu-
Acidic clusters are important for activity of both basolateral sorting determinants of the LDL-R. The importance of acidic clusters for activity of the distal and proximal basolateral sorting determinants was assessed by substituting each acidic residue with an alanine or by COOH-terminal deletion. In A, the role of the acidic cluster in the distal determinant was tested using a receptor whose proximal determinant was also mutated in tyrosine at position 18. In B, the role of the acidic cluster in the proximal determinant was tested using receptors whose distal determinants were removed by deletion. In each case, the cell surface distribution of wild-type and mutant LDL-Rs in stably transfected MDCK cells was determined by binding $^{125}$I-C7 IgG to either the apical or basolateral cell surface at 4°C. After removal of unbound antibody, filters were excised and radioactivity was determined in a gamma counter. Values are given as the percent of total cell surface receptors (total binding was between 10,000 and 100,000 cpm) and represent mean values ± 1 SD of at least three independent experiments performed in duplicates. For experiments shown in B, only clones with an expression level of less than 50,000 receptors were used. The asterisks denote mutants which have been previously published, although the actual data shown here are new. C shows that removal of the three acidic amino acids from either the distal (upper panels) or proximal (lower panels) determinants greatly increases the fraction of newly synthesized receptor that appears at the apical surface. The data for wild-type LDL-R and the other mutants shown in panels A and B have been published previously (Matter et al., 1993). Newly synthesized wild-type LDL-R was never detected at the apical surface. Transfected cells were plated on filters, pulse-labeled with $[^{35}S]$methionine/cysteine and chased for the indicated periods of time. After cooling on ice, either the apical (A) or basolateral (B) cell surface was biotinylated and, then, the cells were extracted. LDL-R were immunoprecipitated and eluted from the immunobeads by boiling in SDS. One fourth of the sample was used for determining total labeled receptors and the rest was diluted and reprecipitated with streptavidin-agarose. The samples were analyzed by SDS-PAGE and fluorography. Fluorographs were quantified by image digitization, and values for recovered surface receptors were normalized by total immunoprecipitable receptors.

Glycine 34 Is Also Important for Activity of the Distal Basolateral Targeting Determinant

The only other membrane protein with a basolateral sorting signal unrelated to coated pit localization domains which has
been analyzed in any detail is the rabbit pIg-R (Casanova et al., 1991). The pIg-R signal contains a tyrosine on which it appears to be only weakly dependent for activity (Aroeti et al., 1993). Moreover, the limited sequence homology between the pIg-R signal and the distal signal of LDL-R does not involve residues required for basolateral targeting of LDL-R (Matter et al., 1992; Yokode et al., 1992). To determine whether any relationship between the pIg-R and LDL-R signals exists and if other residues in the distal LDL-R signal were important to its activity, we sequentially substituted alanines for each amino acid residue surrounding the critical tyrosine at position 35 (Fig. 1 A). The mutations were introduced into an LDL-R whose proximal basolateral targeting signal was inactivated by a tyrosine to alanine mutation at position 18 (Y-A18); thus, only the activity of the distal signal was monitored. In previous work, we had already found that alanine substitutions for serine 36, tyrosine 37, and arginine 40 had no effect on basolateral targeting (Matter et al., 1992).

Fig. 3 illustrates that only one of the substitutions had a significant effect on the performance of the distal basolateral sorting signal. The substitution of the glycine adjacent to tyrosine 35 (YG-A18A34) resulted in a receptor with a markedly increased apical expression (∼55%) and also affected sorting of newly synthesized receptors in the Golgi complex (Fig. 3 B). The inhibition of sorting caused by this mutation was only slightly weaker than the tyrosine to alanine substitution at position 35 (∼70% apical) (Matter et al., 1992). Quantitatively similar results were obtained if glycine 34 was replaced by long chain aliphatic or charged amino acids (isoleucine, glutamic acid, or arginine; not shown), suggesting that a glycine may be specifically required at this position. Thus, at least one key characteristic of the distal basolateral sorting determinant of the LDL-R is a glycine-tyrosine motif followed by the 1–3 downstream negatively charged amino acids.

The Proximal Basolateral Targeting and Endocytosis Determinants Have Distinct Structural Requirements

Even though the proximal basolateral targeting determinant and the signal for coated pit localization both depend on tyrosine 18 for activity, they can be distinguished at least by the fact that the basolateral sorting involves COOH-terminal acidic residues not required for endocytosis. To better define the structural relationship between the two signals, we analyzed the effects of several point mutations around the tyrosine at position 18. Of particular interest were asparagine 15 and proline 16 since both of these had been shown to be important for endocytosis, as part of the canonical NPXY tetrapeptide motif for coated pit localization (Chen et al., 1990; Trowbridge, 1991). The mutations were introduced into a truncated LDL-R mutant (CT27; Fig. 1 A) which contains only the proximal basolateral targeting determinant and lacks the distal signal. Mutant receptors were transfected into MDCK cells and polarity was assayed as usual. Clones with low and high expression levels were analyzed since the proximal basolateral targeting signal has only a low capacity (Matter et al., 1992).

Fig. 4 A shows the fraction of total cell surface receptors that was expressed on the apical plasma membrane as a function of expression level. As shown previously, the CT27 dele-
with increasing expression levels, a greater fraction of the receptor was found on the apical surface (∼70% at 2 × 10^6 receptors/cell) (Matter et al., 1992). Changing the valine at position 17 to isoleucine was without effect. Although a conservative mutation, this result suggested that the precise residue NH2 terminally adjacent to the critical tyrosine is not critical, as is true for the coated pit localization signal. In contrast, converting the COOH terminally adjacent glutamine 19 to a serine (CT27Q-S19) significantly reduced the efficiency of basolateral transport at low expression levels. Mutation of this residue had no effect on LDL-R endocytosis in transfected fibroblasts (Davis et al., 1987) or MDCK cells (not shown).

We next analyzed the effect of point mutations of asparagine 15 (CT27N-A15) and proline 16 (CT27P-A16). As expected from observations using transfected fibroblasts (Chen et al., 1990), both mutations greatly reduced internalization of LDL-R in MDCK cells (Fig. 4 B). However, not only did these mutations fail to reduce basolateral sorting, they actually enhanced it. Substituting an alanine for asparagine 15 increased the efficiency of basolateral sorting such that higher levels of LDL-R expression were required before the CT27N-A15 mutant began to appear at the apical surface (Fig. 4 A). Even more interesting was the CT27P-A16 mutant, in which the proline at position 16 was replaced by an alanine. In this case, the mutant receptor was expressed 90–95% at the basolateral surface irrespective of receptor expression level (Fig. 4 A). Thus, the point mutation in proline 16 rendered the proximal sorting determinant into a signal with a capacity similar to the distal determinant. Cell surface insertion experiments indicated that this reflected an increased capacity for basolateral sorting of newly synthesized receptors in the Golgi complex (Fig. 4 C). Thus, apart from a common dependence on the tyrosine at position 18, the proximal basolateral targeting signal of LDL-R appears to be largely distinct from the receptor's coated pit localization domain. At least two amino acids (proline 16 and asparagine 15) are required for endocytosis but not basolateral targeting. In contrast, glutamine 19 is important for activity of the proximal basolateral sorting determinant but not for endocytosis.

**Figure 4.** Structural requirements for basolateral targeting and endocytosis. (A) Cell surface distribution as a function of expression level of wild-type and mutant LDL-R was assessed by binding 125I-C7 IgG to the apical or basolateral membrane of MDCK cells expressing different amounts of receptors. To be able to analyze a greater spectrum of expression levels, expression was further enhanced in some cell lines by induction with sodium butyrate (10 mM, overnight). This treatment does not affect the integrity and polarity of MDCK cells (Matter et al., 1992). The percent of total cell surface receptors in the apical membrane is given as a function of total cell surface receptors per cell. Values for total 125I-C7 IgG binding in cpm were between 10,000 and 100,000 cpm. (B) Internalization was determined by binding 125I-C7 IgG on ice to MDCK cells expressing wild-type or mutant LDL-R. After removing unbound antibody, cells were incubated at 37°C for the indicated time intervals. Internalization was determined by removing remaining surface-bound antibody by acid wash on ice. Internalized antibody was plotted at each time point as the percentage of antibody bound at time zero (9,000–13,000 cpm). Points represent determinations in triplicate that differed by <19%. (C) The removal of the proline at position 16 blocked endocytosis and increased the fraction of receptor found at the basolateral surface. C shows that, even at high levels of receptor expression (1.3 × 10^6 receptors/cell), newly synthesized CT27P-A16 was inserted almost entirely into the basolateral plasma membrane confirming that this mutation enhanced basolateral sorting in the TGN. See the legend to Fig. 2 for details. The asterisks denote mutants which have been previously published, although the actual data shown here are new.
Phenylalanine Can Substitute for Tyrosine 35 But Not for Tyrosine 18

Many signals for coated pit localization and basolateral targeting depend on critical tyrosines for activity. In most cases, the tyrosine can be replaced by another aromatic residue (phenylalanine) suggesting that the tyrosine's hydroxyl group is not essential (Davis et al., 1987; Trowbridge, 1991). In the case of human lysosomal acid phosphatase (LAP), however, a tyrosine to phenylalanine mutation was found to reduce endocytosis in MDCK cells (Prill et al., 1993). To further define the structural relationships between the two basolateral sorting and internalization signals in LDL-R, we next determined the effects of phenylalanine substitutions on the activities of the proximal and distal determinants. As above, substitutions of tyrosine 35 were introduced into a mutant LDL-R with an inactivated proximal determinant (Y-A18) and those of tyrosine 18 into a mutant which lacked the distal determinant (CT27).

As found previously, replacement of tyrosine 35 with an alanine (YY-A18A35) largely inactivated the distal basolateral sorting determinant (Matter et al., 1992). If tyrosine 35 was changed to a phenylalanine (YY-A18F35), however, basolateral expression of the receptor was not affected (Fig. 5 A). In contrast, substitution by serine (YY-A18S35), strongly disrupted basolateral transport although less dramatically than the tyrosine to alanine mutation. Thus, position 35 requires an aromatic amino acid, and not just a hydroxyl-containing residue, for proper functioning of the distal basolateral targeting determinant.

Fig. 5 B illustrates the effect of phenylalanine and serine substitutions at position 18 on the activity of the proximal determinant. Since the proximal determinant has only a low capacity for basolateral sorting, only cell lines with low expression levels were analyzed (<4 x 10^4 receptors/cell). As demonstrated previously, changing tyrosine to alanine (CT27Y-A18) completely inactivated the proximal basolateral sorting determinant. Unlike the distal determinant, however, a phenylalanine was unable to substitute for tyrosine 18 (CT27Y-F18). Changing the tyrosine to a serine (CT27Y-S18) also completely inactivated the signal. Therefore, the proximal determinant appears to require a tyrosine at position 18 since it cannot be exchanged with another aromatic or hydroxylated amino acid. Thus, the two basolateral targeting signals of the LDL-R do not only have different capacities but exhibit also different degrees of tyrosine dependence.

LDL-R expressing a tyrosine to phenylalanine mutation at position 18 is capable of rapid endocytosis when expressed in CHO cells (Davis et al., 1987). Thus, the failure of phenylalanine to substitute for the tyrosine in the proximal basolateral targeting may represent yet another difference in the structural requirements for endocytosis and basolateral transport. Therefore, we determined the rates of endocytosis exhibited by mutant LDL-Rs in transfected MDCK cells by measuring the rate of internalization of prebound ^125I-labeled anti-LDL-R antibody C7 (Matter et al., 1992).

Fig. 5 C shows that, as expected, endocytosis was slowed in receptors bearing alanine or serine substitutions in place of tyrosine 18. Surprisingly, however, the phenylalanine to tyrosine substitution inhibited endocytosis as efficiently as the alanine substitution. It is not clear whether this result reflected differences in cell type, species, or the background into which the mutation had been introduced (CT27 deletion mutant vs full-length LDL-R). Nevertheless, both rapid internalization and basolateral targeting via the proximal determinant exhibited a strict tyrosine dependence in MDCK cells.

Shortening the LDL-R Cytoplasmic Domain Alters Sequence Requirements for Basolateral Targeting

The proximal and distal basolateral signals in LDL-R are related by common sequence features: requirement for a critical tyrosine followed by a cluster of negatively charged residues. While this general arrangement can be found in the cytoplasmic domains of many receptors known to reach the
basolateral surface of MDCK cells (see Discussion), several basolateral proteins are known to exhibit a tyrosine dependence for polarized transport but have relatively short cytoplasmic tails (<15 residues) which lack COOH-terminal acidic residues. Examples include a tyrosine-containing influenza virus hemagglutinin mutant (Brewer and Roth, 1991) and VSV G protein (Thomas et al., 1993). We asked whether these signals were fundamentally different from those found in LDL-R by determining whether shortening the LDL-R cytoplasmic tail removed the requirement for acidic residues in basolateral targeting.

This possibility was tested for the LDL-R distal domain which normally exhibits a strong dependence on its downstream acidic cluster in the full length receptor tail (Fig. 2 A). A receptor mutant bearing a large internal deletion that removed residues 5-31 (Δ5-31; Fig. 1 A) was efficiently transported to the basolateral surface (<5% apical) despite the fact that the critical tyrosine residue was translocated to a position 8 residues from the presumptive membrane insertion site (Matter et al., 1992) (Fig. 6). Removing the COOH-terminal acidic cluster by deleting the last 8 residues from the internally truncated receptor reduced basolateral expression (~65% basolateral; Δ5-31CT42) (Fig. 6). However, this mutation preserved more basolateral targeting activity than when the same 8 residues were deleted from a full length receptor tail (<10% basolateral; CT42Y-A18) (Fig. 2 A). Identical results were obtained if this region of the LDL-R cytoplasmic domain (residues 32-42; Fig. 1 A) were transferred to the extracellular domain and membrane anchor of mouse FcRII (Fig. 6). Thus, shortening the LDL-R cytoplasmic tail from 50 to 15 residues and moving the distal determinant to a site more closely apposed to the membrane reduced the dependence of the distal determinant on its cluster of three acidic amino acids. This suggests that basolateral sorting determinants in proteins with long and short cytoplasmic domains are, in fact, related.

A Di-Leucine Motif Is Important for Endocytosis and Basolateral Transport of FcRII-B2

Although both basolateral sorting determinants in LDL-R are clearly distinct from coated pit localization signals, both depend on tyrosine residues. This raises the issue of how receptors which do not have tyrosine-dependent internalization signals can reach the basolateral surface of MDCK cells. One well documented example of such a receptor is the mouse IgG Fc receptor FcRII-B2. We have previously found that the only tyrosine in its coated pit localization domain can be changed to an alanine without a marked reduction in the rate of endocytosis in transfected CHO cells (Miettinen et al., 1992). Additionally, the FcRII-B2 basolateral targeting signal resides within the same region of the cytoplasmic domain (residues 18-31) that is required for rapid endocytosis (Hunziker and Mellman, 1989; Hunziker et al., 1991).

To define the amino acid residues in the cytoplasmic tail of FcRII-B2 minimally required for endocytosis and basolateral sorting, we constructed chimeric receptors between FcRII-B2 and LDL-R (Fig. 1 B). Sequences comprising FcRII-B2 cytoplasmic domain residues 18-31 or 22-31 were spliced onto the COOH terminus of a truncated LDL-R (CT33Y-A18) that mediates neither basolateral sorting nor endocytosis (Matter et al., 1992). The chimera were stably expressed in MDCK cells and analyzed for polarity of expression and endocytosis.

The capacity of the chimera to mediate endocytosis was first measured by monitoring the internalization of 125I-labeled C7 IgG. After 1 h at 37°C, the cells were cooled on ice, washed at neutral pH to remove unbound antibody, and then surface-bound antibody eluted by low pH. The ratio of internalized vs total bound antibody was calculated and expressed as percent of the value obtained for cells expressing wild-type LDL-R. As shown in Fig. 7 A, both chimera (LFCR18-13 and LFCR22-31) were able to internalize antibody, albeit 70-75% as efficient as wild-type LDL-R. Cells expressing the LDL-R mutant from which the chimera were constructed (LDL-R: CT33Y-A18) were far less able to mediate internalization of 125I-C7 (30% of wild-type LDL-R). Thus, amino acids 22-31 of the cytoplasmic domain of FcRII-B2 were sufficient for endocytosis.

The polarity of expression was analyzed as described above by binding the LDL-R-specific antibody C7 to either the apical or basolateral cell surface of transfected MDCK cells grown on polycarbonate filters. Fig. 7 B shows that both chimera were expressed basolaterally (>90%) while, as expected, CT33Y-A18 was >90% apical. Thus, in addition to endocytosis, amino acids 22-31 of the cytoplasmic domain of FcRII-B2 were necessary and sufficient to mediate basolateral targeting of an apical protein.

While residues 22-31 contain a tyrosine at position 26, it is not required for FcRII-B2 localization at clathrin-coated pits in transfected fibroblasts (Miettinen et al., 1992). This region also contains adjacent leucines (positions 28 and 29) which have recently been found to comprise a motif that creates a tyrosine-independent coated pit localization domain in at least three other receptors (Letourneur and Klausner, 1992; Johnson and Kornfeld, 1992a,b). To determine which of these motifs are active in MDCK cells, we next substituted the tyrosine, either one or both of the two leucines, or all three amino acids with alanines. For convenience, the point
Mutations were generated using a COOH terminally truncated FcRII-B2 (CT31) which is capable of mediating both endocytosis and basolateral sorting in MDCK cells (Hunziker et al., 1992). Thus, the two leucines are critical for endocytosis of FcRII-B2.

To determine whether a tyrosine or di-leucine motif was critical for basolateral sorting of FcRII-B2, we grew transfected cells on Costar Transwell filters and measured the polarity of expression by binding 125I-labeled Fab fragments of the anti-FcRII antibody 2.4G2 (Matter et al., 1992) (Fig. 8 B). As described previously, the CT31 was detected primarily (80%) at the basolateral surface (Hunziker et al., 1991). Even though the tyrosine to alanine substitution (CT31Y-A26) reduced the relative amount of basolateral receptor (~60%), it did not completely inactivate the determinant. In contrast, substitution of the two leucines (CT31L-A28A29) resulted in a receptor which was predominantly expressed on the apical surface (~80%); removal of tyrosine 26 (CT31YLL-A26A28A29) did not enhance this phenotype. As for their effects on endocytosis, single substitutions of either leucine 28 (CT31L-A28) or 29 (CT31L-A29) also led to a strong inactivation of the sorting determinant, only slightly less complete than the double leucine mutation. Thus, the di-leucine motif is not only required for endocytosis but also for basolateral targeting of FcRII-B2.

To ascertain whether the leucine to alanine substitutions altered sorting in the Golgi complex as opposed to receptor redistribution by transcytosis, we monitored cell surface insertion of newly synthesized FcRII. Transfected MDCK cells were metabolically labeled, and then chased for various periods of time. The cells were then placed on ice and the apical and basolateral surfaces selectively biotinylated. Biotinylated receptors were isolated and analyzed by SDS-PAGE. Fig. 8 C shows that the truncated FcRII-CT31 was transported directly to the basolateral surface. Conversely, the leucine to alanine double mutant (CT31YLL-A28A29) appeared directly at the apical surface. Thus, the di-leucine motif is required for the polarized sorting of newly synthesized FcRII, presumably in the TGN.

Finally, it was of interest to determine the extent to which the endocytosis and basolateral targeting determinants were related apart from their common dependence on the di-leucine motif. For this purpose, we performed an alanine scan of the amino acids between position 22-31. Polarity and endocytosis was assayed as described above and the data directly compared. As summarized in Fig. 9, each mutation had very similar effects on both basolateral expression and endocytosis. Substitutions at positions 22 to 26 partially reduced the efficiency of both while, interestingly, replacement of the amino acids directly adjacent to the di-leucine motif was without any effect. Substitutions of either one of the two leucines resulted in the strongest inhibition of both signals. Thus, in contrast to the tyrosine-dependent proximal determinant of LDL-R, the structural requirements for basolateral sorting and endocytosis mediated by the tyrosine-independent determinants of FcRII-B2 appear to be strikingly similar.

**Discussion**

**Common Motifs in the Basolateral Targeting Determinants of the LDL-R**

We have previously identified two targeting signals in the cytoplasmic domain of the LDL-R (Matter et al., 1992). A signal with a low capacity for basolateral transport was found in a membrane-proximal position overlapping with the clathrin-coated pit domain, and a high capacity determinant was localized in the COOH-terminal half of the cytoplasmic domain. Both of these determinants were shown to depend on a tyrosine for activity. We have now found that tyrosine dependence is not the only feature common to the two signals: both contain clusters of three acidic amino acids at sites several residues downstream from each tyrosine. The activ
Figure 8. Endocytosis and cell surface expression of mutant FcRII-B2. (A) FcRII-B2 deletion mutant (CT31) bearing a series of point mutations affecting tyrosine 26, leucine 28, and/or leucine 29 were monitored for their ability to mediate endocytosis. Ligand internalization was determined by binding preformed IgG-HRP complexes to cells expressing mutant Fc receptors. After incubating the cells at 37°C for the indicated intervals of time, cells were cooled down and HRP-activity was measured with (total bound ligand) or without (ligand still at the cell surface) detergent. Internalization was calculated by subtracting the latter from the first value and expressed as percent of total bound HRP. Shown are mean values of two experiments performed in duplicate. (B) Cell surface distribution was determined by binding [125I]Fab fragments derived from the anti-Fc receptor antibody 2.4G2 to either the apical or basolateral cell surface as described above for anti-LDL-R antibodies. Shown are mean values ± 1 SD of at least three different experiments with cell lines expressing between 80,000 and 300,000 receptors per cell. (C) Cell surface insertion of newly synthesized receptors was monitored by cell surface biotinylation. Transfected cells were metabolically labeled for 15 min, and then chased for the indicated periods of time. After cooling on ice, either the apical (A) or basolateral (B) cell surface was biotinylated and, then, the cells were extracted. Fc receptors were immunoprecipitated and eluted from the immunobeads by boiling in SDS. One fourth of the sample was used for determining total-labeled receptors and the rest was diluted and reprecipitated with streptavidin-agarose. The samples were analyzed by SDS-PAGE and fluorography. Fluorographs were quantified by densitometric computer-assisted digitization, and values for recovered surface receptors were normalized by total immunoprecipitable Fc receptors.
motif suggests that the two basolateral targeting signals in adjacent to each tyrosine, although neither the identity nor position of this amino acid was conserved. Nevertheless, the common dependence on a single amino acid LD-L-R are structurally related, then differentiating basolateral concerning the nature of the basolateral determinants, this finding has several important implications.

If the proximal and distal basolateral targeting signals in LDL-R are structurally related, then differentiating basolateral signals on the basis of their relationship to signals for accumulation at clathrin-coated pits may be simply fortuitous. Indeed, in the case of the proximal signal, neither the asparagine nor proline which constitute important elements of the NPXY-type coated pit localization domain found in LDL-R are required for basolateral transport. In other words, apart from sharing a common tyrosine, the proximal signal bears no structural relationship to the coated pit localization signal.

We had also distinguished between the proximal and distal basolateral targeting determinants on the basis of their different capacities for basolateral sorting (Matter et al., 1992). At increasing levels of LDL-R expression, the proximal determinant became progressively less efficient at directing basolateral transport as if the sorting apparatus responsible for its recognition became saturated. We now find that by simply replacing the proline at position 16, the capacity of the proximal determinant became indistinguishable from the distal determinant.

Together, these considerations imply that clathrin or clathrin adapters are not directly involved in recognizing the proximal basolateral targeting determinant in LDL-R. It remains possible, however, that coated pit and basolateral signals were derived from each other during evolution and do have some structural features in common that are not obvious from primary sequence. They thus may interact with related families of adapter-like proteins in the cytosol. Conceivably, a second distal signal arose in LDL-R to increase the basolateral sorting capacity over what was possible due to the dual-function tyrosine in the proximal determinant.

**Pathways of Transport to the Basolateral Surface of MDCK Cells**

The precise pathway, or pathways, leading from the TGN to the basolateral surface of MDCK cells remains a critical unknown. Presumably, sorting in the TGN partitions newly synthesized membrane and secretory proteins into at least three distinct classes of transport vesicles: one leading to the basolateral surface, one to the apical surface, and a third to endosomes and lysosomes (Wandinger-Ness et al., 1990; Kornfeld and Mellman, 1989). While the endosomal pathway is thought to be uniquely targeted by the formation of clathrin-coated vesicles from the TGN, it is conceivable that a single pathway leads to endosomes which actually represents the final site of sorting, although evidence for such a mechanism is lacking.

In any event, it is not certain whether there is a single pathway or multiple classes of vesicles (emanating from the TGN and/or endosomes) that are responsible for basolateral transport. The existence of two or more distinct classes of basolateral signals would suggest that at least two pathways might exist. Since it now seems possible that, at least for LDL-R, the coated pit-related and -unrelated signals may reflect a single type of determinant, it is more likely that only a single pathway exists entry into which is specified by a single class of basolateral signal. It is still possible, however, that any protein leaving the TGN of MDCK cells via the clathrin-mediated endosomal pathway would reach the basolateral surface by an indirect route. Thus, irrespective of whether the basolateral or coated pit signals were recognized in a given protein, the end result would be the same. Since both apical and basolateral early endosomes in MDCK cells are capable of mediating signal-dependent recycling to the basolateral surface (Matter et al., 1993), they would be able to similarly target newly synthesized proteins delivered from the TGN.

Our finding that a common di-leucine motif directs both endocytosis and basolateral transport of FeR-II-B2 is not necessarily inconsistent with the possibility that only a single class of basolateral signal exists. Although the di-leucine motif is, at least at the level of amino acid sequence, quite distinct from any of the more common tyrosine/phenylalanine-dependent coated pit localization signals, proteins containing either type of signal are likely to accumulate at the same clathrin-coated pits and are functionally indistinguishable. It is possible that coated pits contain adapter proteins that have specificities for entirely distinct types of localization signals. However, a simpler explanation is that di-leucine and tyrosine-containing motifs form similar secondary or tertiary structures that serve as the basis for their recognition by a common sorting system in clathrin-coated pits.

At present, tyrosine-containing coated pit localization domains are thought on the basis of structural predictions and two-dimensional NMR of peptides in solution to assume a reverse β-turn configuration displaying the critical tyrosine at an exposed site in the loop (Trowbridge, 1991; Vaux, 1992). The hydrophobic surface presented by this arrange-
ment might, in some sequence contexts, be mimicked by two adjacent leucines or other small hydrophobic residues. It is this feature that may underlie the common dependence of even distinct recognition systems on tyrosines and di-leucines. Clearly, other sequence elements must contribute to the specificity of these recognition events.

In some contexts, a single signal may be recognized by more than one sorting system. Thus, some—but apparently not all—tyrosine-containing determinants can be recognized not only by coated pits on the plasma membrane but possibly also in the TGN. Although direct evidence for clathrin localization in the Golgi complex is lacking, the lgp family of lysosomal proteins contains a conserved glycine-tyrosine motif that not only mediates sorting directly from the TGN to endosomes but also directs rapid-coated pit–dependent endocytosis at the cell surface (Williams and Fukuda, 1990; Harter and Mellman, 1992). The di-leucine motifs in the cytoplasmic tails of CD3 and LIMP-2 appear to function in much the same way (Letourneur and Klausner, 1992; Vega et al., 1991; Ogata and Fukuda, 1994). Thus, it is possible that in the absence of clathrin adapters, coated pit signals may be recognized, albeit less well, by the basolateral sorting machinery. Such a situation may explain why the LDL-R proximal domain specifies only a limited capacity for basolateral sorting in the TGN. Upon inactivation of its coated pit localization activity by removal of proline 16, it can now be recognized only as an "authentic" basolateral targeting determinant.

**Generality of Basolateral Sorting Signals**

Given the high degree of variability even among the far more extensively studied signals for coated pit localization, it is premature to propose a common motif (or motifs) characteristic of all basolateral targeting determinants. However, it is interesting to note that clusters of two or more acidic amino acids downstream from tyrosine or phenylalanine (or di-leucine) residues are found in the cytoplasmic domains of many proteins known to reach the basolateral surface of MDCK cells. These include E-cadherin (Nagafuchi et al., 1987; Ringwald et al., 1987), transferrin receptor (McClelland, 1984), cation-independent and -dependent mannose-6-phosphate receptors (Lobel et al., 1988; Oshima et al., 1988; Pohlmann et al., 1987), LAP (Pohlmann et al., 1988), plg-R (Mostov et al., 1984), and FcRII-B2 (Lewis et al., 1986). It is either not yet known or not clear that this motif plays a role in basolateral targeting of these proteins. However, as demonstrated for LDL-R, conclusions are at present limited by the facts that any one cytoplasmic tail may contain redundant targeting signals, that expression level can influence apparent sorting efficiency, and that cytoplasmic tail length can influence whether the acidic cluster forms an essential part of the signal.

In addition to LDL-R and FcRII-B2, the only other proteins whose basolateral targeting determinants have yet been analyzed in any detail are LAP and plg-R (Prill et al., 1993; Areoti et al., 1993). The LAP signal is similar to the LDL-R proximal signal in that it clearly contains a tyrosine which is shared by its coated pit localization domain. It is also followed by downstream acidic residues, only one of which is partly required for both basolateral targeting and endocytosis (Prill et al., 1993). Although the downstream acidic clusters in LDL-R are not required for endocytosis, we do not yet know whether one or all of the residues within each cluster are equally important for basolateral targeting. The LAP signal also contains two proline residues two and three positions upstream from the tyrosine (PPGY), and, unlike the LDL-R proximal determinant, an alanine substitution of the second proline blocks both internalization and polarized sorting. Like LDL-R, however, the coated pit and basolateral determinants are not identical since phenylalanine will substitute for the tyrosine for basolateral targeting but not for endocytosis. The magnitude of the effects observed due to the mutations at these residues are comparable to those we have observed for LDL-R. It should also be mentioned, however, that the presence of a proline two positions upstream from a tyrosine is not an invariant feature of either coated pit localization domains or of basolateral targeting signals. Neither plg20 nor hemagglutinin-Y453 contain prolines in this position (Fig. 10).

Figure 10. Amino acid sequences of identified basolateral sorting determinants. The amino acid sequences of basolateral targeting determinants are shown in the single letter code. Amino acids known to be critical (<30% basolateral expression) are printed in bold and highlighted in dark gray boxes, those of moderate importance are highlighted in light gray circles. All other residues have either been found not to affect basolateral transport (LDL-R, LAP, plg-R) or have not been analyzed. Sequences and data for lysosomal acid phosphatase (LAP) were taken from Prill et al. (1993), for plg20 from Hunziker et al. (1991), for hemagglutinin-Y453 from Brewer and Roth (1991), for asialoglycoprotein receptor (ASGP-R) H1 subunit from Geffen et al. (1993), for plg-R from Areoti et al. (1993), and for vesicular stomatitis virus G protein (VSV-G) from Thomas et al. (1993). In the case of VSV-G protein, removal of the tyrosine is as effective at blocking basolateral sorting as a deletion of the entire cytoplasmic domain. Since either of these mutations only reduced basolateral expression to 50%, VSV-G protein can reach the basolateral surface using a mechanism that does not absolutely require its cytoplasmic domain (perhaps by binding to its cognate receptor in the TGN). Nevertheless, the VSV-G protein signal is classified as exhibiting a "weak" tyrosine dependence. Similarly, it was difficult to classify the importance of individual residues in plg-R since the effects of point mutations were modest relative to those obtained for other receptors (Areoti et al., 1993).
In addition to the distal determinant of LDL-R, the only other coated pit-unrelated basolateral signal defined thus far has been in the cytoplasmic tail of the plg-R (Casanova et al., 1991). Its relationship to the LDL-R signal, or any other signal, remains unclear. It was first identified as being contained within a fortuitous restriction fragment which could cause basolateral transport when spliced onto a GPI-anchored apical protein. In contrast to the distal signal of the LDL-R, the plg-R determinant is in a membrane-proximal position and seems to exhibit only a weak tyrosine dependence (Aroeti et al., 1993). The possible involvement of downstream (but not clustered) acidic residues has not been evaluated in the full length receptor. The plg-R tyrosine does not contain an adjacent glycine, but several other residues were important for maximal basolateral targeting activity (Aroeti et al., 1993). These residues are either absent or in the “wrong” positions and thus not required in the LDL-R distal determinant (see Fig. 10, below). However, the effects of these mutations on basolateral transport of the plg-R were all very modest (Aroeti et al., 1993) thus limiting any comparisons. Since the plg-R is a protein highly specialized for basolateral to apical transcytosis, its basolateral targeting domain may be modified to facilitate its inactivation by ligand binding or phosphorylation upon reaching the basolateral surface (Casanova et al., 1990; Hirt et al., 1993).

In Fig. 10, we have summarized the amino acid sequences of known or presumptive basolateral targeting signals found in several proteins. A comparison of tyrosine-dependent signals fails to yield any obvious similarities; but at this stage, a similar comparison of coated pit signals would also reveal little in the way of common features. Nevertheless, a few interesting features emerge. The distal determinant of the LDL-R shares a glycine-tyrosine-motif with Igpl20 and LAP, both of which are “coated pit-related”. For activity of the LDL-R signal, this glycine is important while basolateral targeting of Igpl20 and LAP is either slightly or not affected by an alanine substitution at this position (Hunziker et al., 1991; Prill et al., 1993). The glycine is not conserved in the coated pit related proximal determinant of LDL-R; in this case, the COOH terminally adjacent glutamine is important. Similarly, the hemagglutinin mutant lacks a glycine but the tyrosine is followed by an arginine which is known to be of some importance in the LAP basolateral targeting determinant. The importance of the arginine in hemagglutinin has not yet been investigated. In the distal signal of the LDL-R, the position following the tyrosine is neither a glutamine nor an arginine (but a serine) and is not important for activity. Even though there is no additional amino acid conserved in all the known tyrosine-dependent determinants, the tyrosine often appears to be preceded by a glycine and followed by an arginine or glutamine. Additionally, the amino acid in either one of these two positions appears to influence the importance of the other position. Thus, if a glycine is in front of the tyrosine, the other position is of lesser importance (e.g., the distal determinant of the LDL-R). In contrast, if the tyrosine is followed by a glutamine or arginine, a glycine in this position is not very important (e.g., lysosomal acid phosphatase, proximal determinant of LDL-R, hemagglutinin-Y543). The presence of a glycine may even modify the importance of the tyrosine. In the distal determinant of the LDL-R and the basolateral sorting signal of LAP (where the tyrosine is preceded by a glycine), the tyrosine can be changed to a phenylalanine without a significant loss in activity. In the proximal determinant of the LDL-R, which has a valine instead of a glycine, a phenylalanine cannot substitute for the tyrosine. If a determinant has a glycine as well as an arginine in the two positions, a proline two positions upstream from the critical tyrosine may no longer confer low capacity (distal determinant of the LDL-R) but may even improve the activity of a determinant (lysosomal acid phosphatase). Furthermore, this (G)-Y-(Q/R) motif can be followed by acidic amino acids which are of importance in the context of longer cytoplasmic tails. It is clear that these “rules” are very speculative, but they can be tested in known basolateral sorting determinants and applied to help organize the search for such signals in other basolateral membrane proteins.

It is far too early to determine whether predictable or common features exist that define a single, or multiple, classes of basolateral targeting determinants or to use this information for structural studies. These initial efforts to do so indicate that this problem is likely to recapitulate the search for signals involved in coated pit localization. More importantly, the identification of any specific sequences involved in basolateral sorting has demonstrated the likely existence of cytosolic proteins that decode these sequences to facilitate polarized transport. Defining mutations that prevent the recognition of these sequences will, in turn, facilitate the identification and characterization of the sorting machinery itself.

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