Clathrin-coated Pit-mediated Receptor Internalization

ROLE OF INTERNALIZATION SIGNALS AND RECEPTOR MOBILITY*

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Most signals controlling receptor-mediated endocytosis have been identified by alteration of sequences present in receptors normally internalized via clathrin-coated pits. In the present work we have reconsidered the factors that control internalization the other way around: i.e. by introducing potential internalization sequences in complement receptor 1 (CR1) which does not preferentially associate with clathrin-coated pits.

The analysis of the internalization efficiency of NPxY related motifs generated by substituting His2010 and/or Glu2018 by either Phe or Tyr indicates that FxNPxY is the stronger promoter of endocytosis and that the signal efficiency depends on the presence of aromatic residues (including a tyrosine) at both ends of the -xNPx- motif. Moreover, CR1-tyr (substitution of Glu2018 for Tyr) internalization was superposable to that of a receptor composed of the extracellular and transmembrane domains of CR1 fused to the intracytoplasmic tail of the low density lipoprotein (LDL) receptor (including the FxNPxY motif) (CR1-LDL). When analyzed by fluorescence recovery after photobleaching, the surface mobility of CR1-LDL was decreased as compared with that of CR1-wt, despite a similar association with clathrin-coated pits. The role of receptor mobility in internalization was confirmed by the observation that CR1-tyr, with a deletion of the cytoplasmic tail, was more mobile and more efficiently internalized than CR1-wt.

Receptor-mediated endocytosis allows cells to internalize surface-bound molecules rapidly via specialized domains of the plasma membrane, i.e. clathrin-coated pits (1). The preferential association of receptors with clathrin-coated pits involves peptide motifs of the cytoplasmic tail which interact selectively with proteins associated with these invaginations, presumably the adaptins (2). Several intracytoplasmic internalization signals have been identified so far (3–16). Although heterogeneous, their primary sequence generally includes an aromatic amino acid. Studies showing that transplantation of these sequences into other receptors promotes high efficiency endocytosis suggest that these signals are relatively independent of their context (12). Such comprehensive behavior may originate from their strong propensity to form a type I 3-tum exposing aromatic residues (11, 12, 16, 17).

These internalization signals provide a straightforward explanation for endocytosis efficiency. However other factors involved in the control of receptor internalization via clathrin-coated pits were recently shown to greatly modulate the effects of internalization signals. These include receptor autophosphorylation (18), associated protein tyrosine kinases (19), inhibitory signal sequences intrinsic to the receptor (20), and serine or threonine phosphorylation (21).

Most of the studies dealing with internalization signals have involved the deletion or substitution of amino acid(s) within the cytoplasmic portion of receptors internalized efficiently via clathrin-coated pits. However the interpretation of these studies is rendered difficult by the possible implication of more than one receptor domain in receptor internalization, as exemplified by the FcII receptor (20), and their extension is precluded by the large variety of both receptors and cell types studied. The aim of the present work was therefore to circumvent these potential problems by using a "clathrin-coated pits association neutral" receptor and investigating the relationship between internalization signals and receptor surface mobility in the process of clathrin-coated pits receptor internalization.

We chose the complement receptor type 1 (CR1) for this study. CR1 is a transmembrane glycoprotein (22) with a short cytoplasmic tail devoid of aromatic amino acids. It is neither preferentially associated with nor excluded from clathrin-coated pits in human neutrophils (21).

Using this model, we have reassessed the role of the cytoplasmic tail in general and of the FxNPxY signal sequence in particular. Results obtained demonstrate the fine tuning played by aromatic residues within the FxNPxY sequence in the control of endocytosis, as well as the essential role of receptor mobility in the modulation of receptor-mediated endocytosis.

MATERIALS AND METHODS

Chemicals and Cell Cultures—Cells were grown under 5% CO2 in plastic Petri dishes in Dulbecco's modified Eagle's medium, 10% fetal calf serum for L fibroblasts, and Ham's F-12, 10% fetal calf serum for CHO cells.

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The monoclonal antibody (mAb) 3D9 directed against CR1 has been described previously (23). Fab fragments were produced by papain digestion and purified by ion-exchange chromatography (24). These antibodies were labeled with 125Iodine (Amerham, Buckinghamshire, Great Britain) using IODO-GEN (Pierce) according to the manufacturer's indications. Specific activity ranged from 30 to 30 μCi/μg.

Bovine serum albumin, was purchased from Sigma (Buchs, Switzerland), Taq polymerase from Perkin-Elmer Cetus (Zug, Switzerland), and restriction enzymes from Boehringer Mannheim (Servion, Switzerland) or New England Bio-Labs (Alabisswil, Switzerland). All chemicals used were analytical grade.

Mutagenesis and Cell Transfections—The entire coding sequence of human CR1 (A allele) cloned into the pCDM8 expression vector was first introduced in pABCD by the same procedure as described previously (25) to obtain pCR1-wt with AflII site (in italic) in the LDLR sequence, and a mutated 3' primer pCR1-LDL. A tailless mutant (CR1-tl) was obtained by digesting the pCR1-wt with AflII, SacI, and BglII sites of the CR1 cDNA to generate pCR1-tyr.

To substitute the intracytoplasmic portion of CR1 by that of the LDL receptor, a unique AflII site located at the transmembrane-intracytoplasmic border was first introduced in pABCD by the same procedure as described above and named pCR1-wt. The oligonucleotides used for this mutation (mutated bases are underlined). The two polymerase chain reaction products were then mixed and amplified with the flanking primers CR-A and CR-B, and the fragment subcloned between the AflII, and BglII sites of the CR1 cDNA to generate pCR1-tyr.

Resistance to G418 was provided by cotransfecting the cells with pSV2neo. CR1-positive G418-resistant clones were selected by fluorescence microscopy and confirmed by biochemical analysis. Values obtained after 2 h at 4 °C, a condition in which internalization is blocked, were systematically subtracted from values calculated at all time points at 37 °C. Nonspecific binding (evaluated by adding an excess of mAb to the incubation medium) represented less than 4% of the cell-associated radioactivity in all conditions studied.

Inhibition of coated particle-mediated endocytosis using hypericin was achieved as described previously (31).

FRAP Measurements—Cells grown to confluence on glass coverslips were washed and cooled to 4 °C in Krebs-Ringer phosphate buffer, containing 10 mM glucose, 1 mM Ca2+, and 1 mM Mg2+. Values obtained after 2 h at 4 °C, a condition in which internalization is blocked, were systematically subtracted from values calculated at all time points at 37 °C. Nonspecific binding (evaluated by adding an excess of mAb to the incubation medium) represented less than 4% of the cell-associated radioactivity in all conditions studied.

The equipment used has been described in detail elsewhere (33). The diffusion coefficient (D) of the receptors was calculated according to Axelrod et al. (34) and the percent recovery (R), reflecting the proportion of mobile receptors, was determined according to Jacobson et al. (33). The fluorescence in the bleached spot at time intervals of increasing length (from 1 to 10 s with a final measurement, (Ff), at 60 s) was sampled. The cells were kept at a fixed temperature (14 or 37 °C) during the entire measurements by using a temperature-regulated microscope stage. All measurements were completed within 40–60 min. The equipment used was described in detail elsewhere (33).

RESULTS

Clone Selection and CR1 Expression—Neomycin-resistant transfectants expressing CR1 were selected either by fluorescence-activated cell sorting, using mAb 3D9 and an fluorescein isothiocyanate-labeled anti-mouse IgG or by limiting dilutions and 125I-3D9 binding. Clones expressing approximately the same number of 3D9 binding sites were selected and amplified.

The correct synthesis and surface expression of CR1 constructions were assessed by biosynthetic labeling. In preliminary experiments, various chase periods were analyzed, and CR1 was first detected as pro-CR1 of about 220 kDa, a form that eventually matures to migrate at the expected 250 kD of the A allele of CR1 (35). All CR1 constructs were synthesized correctly and incorporated into the membrane (data not shown).

Validation of CR1-wt as a Clathrin-coated Pits Association

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Validated Genes

[3H]-Methyl thymidine (3H-Me-thymidine) was added and cells incubated further for 2 h at 37 °C. This labeling period was followed by a 3-h chase in F-12 medium with 10% fetal calf serum. Cells were washed three times in cold phosphate-buffered saline and solubilized in 500 µl of lysis buffer (1% Triton X-100 v/v), 50 μM Hepes, 150 mM NaCl, 2 mM phosphate, 1 mM EDTA, 0.1 mg/ml ascorbate, 1 µM leupeptin, pH 7.2) for 30 min on ice. Lysates were cleared at 100 x g for 5 min, and supernatants were incubated overnight at 4 °C with 2 µg of 3D9, and precipitate were recovered by adding 25 µl of protein A-Sepharose (Pharmacia) for 1 h at 4 °C. After five washes, the resin was boiled for 3 min in SDS-sample buffer and the supernatant analyzed on 7.5% SDS-polyacrylamide gel electrophoresis. Gels were stained, dried, and exposed to Kodak XAR film at room temperature.

Resistance to G418 was provided by cotransfecting the cells with ice-cold CHO chemical buffer. Cells were transiently transfected and allowed to incubate for 30 min using fluorescent isothiocyanate-labeled 3D9 at a 1:5 dilution in KRG. The cells were washed in ice-cold KRG, mounted, and inserted into the microscope for FRAP measurements (32, 33). Before the start of the measure-

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Validation of CR1-wt as a Clathrin-coated Pits Association
Neutral Receptor—To verify that in our cell system the short cytoplasmic tail of CR1-wt does not influence its surface localization, we compared the behavior of CR1-wt with that of the tailless form of CR1 (CR1-tl) (Fig. 1). The localization by quantitative EM autoradiography of CR1 tagged with 125I-3D9 showed that CR1-tl association with clathrin-coated pits was undistinguishable from CR1-wt (Fig. 2). By contrast, a chimeric receptor consisting of the external and transmembrane domain of CR1 and the intracytoplasmic tail of the LDL receptor (CR1-LDL), showed a typical preferential association with clathrin-coated pits (Fig. 2). These observations demonstrate that the C-terminal portion of CR1 neither prevents, nor promotes, interaction with clathrin-coated structures.

Role of Internalization Sequences in Receptor Internalization—The presence of a NPKE sequence, 10 amino acids from the transmembrane domain of CR1-wt, allowed us to generate a NPX Y motif by substituting Glu to Tyr at position 2015 (Fig. 1). When CR1-tyr was stably expressed in CHO cells, its internalization was increased as compared with that of CR1-wt (Fig. 3A). Similar results were obtained following transfection in L fibroblasts (data not shown). Thus, in CR1, the presence of a NPX Y internalization motif 10 amino acids from the plasma membrane improved receptor internalization.

As determined by quantitative EM autoradiography, the increased internalization of CR1-tyr was a consequence of an increased association with clathrin-coated pits. Indeed, CR1-tyr association with clathrin-coated pits at 4 °C was more than two times higher than that observed in the case of CR1-wt (Fig. 2), and this value remained constant during incubation at 37 °C (Fig. 3B). The biochemical counterpart of these observations was obtained by measuring internalization in cells incubated in hypertonic medium (0.3 M sucrose), which is known to prevent clathrin-coated pit-mediated internalization (36). Under these conditions, the inhibition of CR1-Tyr internalization was greater than that of CR1-wt, again consistent with an enhanced association of these modified CR1 with clathrin-coated pits (data not shown).

Recently, detailed studies of the internalization motif of the LDL receptor have suggested that: (a) the more efficient sequence is made of an hexapeptide (FxNPxFY) (5, 12), and (b) substitution of the Tyr residue within this sequence by another aromatic amino acid such as Phe or Trp does not affect internalization significantly (9). To determine whether these conclusions applied to our system, and to collect further information on the exact role of the aromatic amino acids in the sequence, we have mutated the aromatic residues at the beginning and the end of the FxNPxFY sequence. Six permutations of Tyr and Phe residues were constructed (Fig. 1), and their endocytic properties were analyzed following transient expression into COS cells. First, the generation a FENPKY (=FxNPxFY) motif 8 amino acids from the transmembrane domain of the molecule (Fig. 1) (CHO-FY) markedly increased the internalization capacity of CR1 as compared not only with that observed in CR1-wt but also with the internalization detected in CR1-tyr (Fig. 4). Second, within the tetrapeptide NPxFY, replacement of Tyr by Phe significantly decreased the efficiency of the internalization signal (Fig. 4). Third, the addition of an aromatic residue at the beginning of the hexapeptide improved internalization except when two Phe are introduced (FxNPxF) (Fig. 4). Fourth, and consistent with previous observations (12), the most potent internalization signal was FxNPxFY.

Thus, the most efficient internalization is obtained when an hexapeptide sequence containing an asparagine and a proline in its middle is flanked at its extremities by aromatic residues, including at least 1 Tyr.

Role of Receptor Mobility in Internalization—The cytoplasmic tail of the LDL receptor contains a FxNPxFY sequence and thus is predicted to promote optimally endocytosis. When a chimeric CR1 bearing the extracellular and transmembrane domains of CR1 and the entire cytoplasmic moiety of

| CR1-wt | KHRKGNHAEVAYAILHISQGGSSVHPRTLQTNENSRVLP-COOH |
| CR1-Tyr | KHRKGNHAEVAYAILHISQGGSSVHPRTLQTNENSRVLP-COOH |
| CR1-LDL | KNWLKNTNENPPYQTEVTHYHCHIQGGSYSPRQMYSLEIVPA-COOH |
| CR1-tl | N-COOH |
| CR1-YY | KHRKGNHYEYVAILHISQGGSSVHPRTLQTNENSRVLP-COOH |
| CR1-FY | KHRKGNFEYEYVAILHISQGGSSVHPRTLQTNENSRVLP-COOH |
| CR1-YF | KHRKGNHYEYVAILHISQGGSSVHPRTLQTNENSRVLP-COOH |
| CR1-FF | KHRKGNFEYEYVAILHISQGGSSVHPRTLQTNENSRVLP-COOH |
| CR1-F | KHRKGNHAEVAYAILHISQGGSSVHPRTLQTNENSRVLP-COOH |

Fig. 1. Schematic representation of the CR1 constructs. TM, transmembrane domain; internalization signals are underlined; point mutations are indicated in boldsface. The intracytoplasmic sequence is given in single-letter amino acid code.
were compared with those of CR1-wt, CR1-tl were internalized (Fig. 1). When the endocytotic properties of these receptors were studied, CR1-tyr was found to be more efficiently internalized than CR1-tyr because of its reduced mobility. However, although increased CR1-tyr internalization correlated with an increase in its diffusion coefficient (D) and mobile fraction (R), the mobility of CR1-wt and CR1-tl showed equivalent values of clathrin-coated pits association (Fig. 2). Such difference could not be ascribed to a difference in clathrin-coated pits association: CR1-wt and CR1-tl showed equivalent values of clathrin-coated pits association (Fig. 2). By contrast, increased CR1-tl internalization correlated with both an increase in its diffusion coefficient (D) and mobile fraction (R) (Fig. 6, B and C). Taken together, these results indicate that lateral mobility plays a key role in the control of receptor internalization.

**DISCUSSION**

In the present work we took advantage of a receptor (CR1) which does not associate preferentially with clathrin-coated pits to determine (a) which mutations of its cytoplasmic domain would confer localization in these specialized surface invaginations and (b) how could receptor lateral mobility modulate this process. Confirming previous observations in polymorphonuclear neutrophils (21), a quantitative EM autoradiographic analysis clearly indicates that, in CHO cells, CR1-wt is neither preferentially associated with nor excluded from clathrin-coated pits and, in this respect, behaves similarly to a tailless form of CR1 (CR1-tl). Thus, CR1 is an appropriate tool to study signals and factors involved in receptor-mediated endocytosis.

A first advantage of a model making use of a clathrin-coated pits association indifferent receptor is that it excludes the possible influence of other domains than the one under study. Potential inhibitory domain(s) such as that recently described in FcII H2 receptor, which actively prevents clathrin-coated pit-mediated receptor internalization (20), indeed hamper(s) the interpretation of studies in which mutation(s) or deletions are introduced into internalization-competent receptors (3-16). A second advantage of our model system is...
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These results confirm the role of the NPxY motif in clathrin-coated pits association of the mutated receptors with clathrin-coated pits. Increased internalization was directly related to an increased stabilization of the wild type form of CR1 up to 3-fold. This implies that the introduction of a tyrosine residue within a NPx context, generating a NPxY motif, increases internalization of the LDL receptor or the insulin receptor compared to the LDL or insulin receptors. These results disagree with previous reports, suggesting that the FxNPxY motif is the complete internalization signal (3, 12). They extend this notion, however, since our data demonstrate that the conditions required to provide the most effective internalization signal is the presence of aromatic amino acids (including at least 1 tyrosine residue) at the extremities of an xNPx context. Moreover, a tyrosine at the end of the sequence is apparently largely responsible for the potency of the signal, and the presence of a tyrosine in the first position can modulate the efficiency of the aromatic residue present at the end. Simple secondary structure analysis using the Chou-Fasman algorithms predicts that a tyrosine in the last position of the tetrapeptide or in the first and last position of the hexapeptide have a stronger propensity than phenylalanine to induce a \( \beta \)-turn conformation (data not shown). Extended investigation of the secondary structure of these signal sequences is necessary to understand fully the mechanisms underlying the observed differences between these peptides.

Although the cytoplasmic tail of the LDL receptor contains a FxNPxY sequence (5, 12) and is known to be efficiently internalized via clathrin-coated pits (1), a chimeric receptor with the extracellular and transmembrane domain of CR1 fused to the complete intracytoplasmic domain of the LDL receptor (containing a FxNPxY sequence) was internalized...
with the same efficiency as CR1-tyr and with only half the efficiency of CR1-FY (containing also a FxNPxY sequence). Since the only segment which differs between CR1-LDL and CR1-FY is the cytoplasmic tail, these results suggest that (an) additional domain(s) within this region contributes to the modulation of LDL receptor endocytosis. On the basis of the observation of the receptors analyzed here by FRAP showing that the LDL receptors are the least mobile, it can be proposed that domains other than the FxNPxY motif affect the lateral mobility of receptors. The length of the cytoplasmic tail is not involved in this process, since the LDL receptor cytoplasmic tail is composed of 49 amino acids, versus 43 amino acids for the CR1 tail. Although clathrin-coated pits could participate in such interactions, they do not appear to play this role since CR1-LDL and CR1-tyr, which display different association with clathrin-coated pits. Based on previous observations demonstrating an interaction of various receptors (i.e. epidermal growth factor receptors, Fc receptors, or insulin receptors) with cytoskeleton elements, a similar possibility could be evoked in the case of the LDL receptor (42–44). Due to the small number of residues of the cytoplasmic domain of LDL receptors, this domain should be amenable to molecular identification using our chimeric CR1.

CR1-tl, which lacks internalization signal, is internalized more efficiently than CR1-wt, probably due to its more rapid lateral mobility; within a given period of time they occupy a larger area of the cell surface than less mobile receptors and hence have an increased probability to be trapped in an invagination undergoing endocytosis. This observation fits with the above-mentioned data regarding CR1-LDL and confirm that the lateral mobility of the receptor interfered with receptor internalization. Another illustration of this effect is provided by studies on the human insulin receptor. We found that the first limiting factor in insulin-induced receptor internalization was its surface redistribution (18). Upon insulin binding, the activation of the endogenous receptor kinase enables it to migrate from the microvilli, where it is initially concentrated, toward non-villous areas of the cells, where clathrin-coated pits are situated (18). These observations highlight both the importance of lateral mobility and internalization signals in the overall process of endocytosis.

In conclusion, the intense study of clathrin-coated pits-mediated internalization has led to the paradigm of signal sequences as mediators of receptor/clathrin-coated pits interaction. Together with other studies, our data confirm the validity of these signals. Our sensitive model system allowed us, however, to extend this concept: it reveals that a fine tuning in the control of endocytosis is carried out by aromatic amino acids. Moreover, it stresses the importance of other regions of the cytoplasmic domain of receptors which may influence phenomena such as their lateral mobility.

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