Molecular Determinants of the Clearance Function of Type C Receptors of Natriuretic Peptides*

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Receptor-mediated endocytosis is the cellular mechanism by which type C receptors of natriuretic peptides exert their clearance function. In the present work, performed in recombinant Chinese hamster ovary cells stably transfected with wild type or mutated human kidney C receptors, we determined net endocytic rates (ER) of C receptor-ligand complexes, lysosomal hydrolysis of ligand (125I-labeled native atrial natriuretic factor, ANF1–28), and receptor recycling. Equilibrium ligand binding, immunocytochemistry, and immunoprecipitation were performed to characterize the transfected receptors. The net ER of recombinant wild type C receptors was 6% of occupied receptors internalized per min, and C receptor-mediated lysosomal hydrolysis of ligand amounted to ~250% of specifically bound 125I-ANF1–28/h, with efficient recycling of internalized C receptors to the cell surface. Hypertonic sucrose reduced net ER and lysosomal hydrolysis of 125I-ANF1–28 more than 10-fold, indicating that endocytosis occurred via clathrin-coated pits. Total deletion of the cytoplasmic domain also reduced net ER and lysosomal hydrolysis of 125I-ANF1–28 by almost 10-fold, whereas deletion of the terminal 28 amino acids of the cytoplasmic tail led to a 4-fold reduction in these parameters. Replacement of cytoplasmic domain Tyr508 by Ala, or Tyr508 and Phe538 by Ala, reduced net endocytosis and lysosomal hydrolysis of 125I-ANF1–28 by 40–50%. Replacement of extracellular domain Cys573 by Ala impeded the constitutive formation of homodimers and reduced by ~50% the net ER and lysosomal hydrolysis of 125I-ANF1–28. These results demonstrate that the cytoplasmic domain of C receptors, Tyr508 within this domain, and constitutive receptor dimerization are the major molecular determinants of the clearance function of C receptors.

Natriuretic peptides, particularly atrial natriuretic factor (ANF), play an important role in volume-pressure homeostasis (1). There are two functionally and biochemically distinct classes of receptors for natriuretic peptides. Guanylyl cyclase receptors (subtypes A and B) are signaling receptors that mediate the biological effects of natriuretic peptides via the generation of cGMP. Clearance (C) receptors play an important role in the removal of natriuretic peptides from the circulation, thus contributing to their plasma homeostasis (1, 2).

C receptors have a very high affinity for all members of the natriuretic peptide family, including ANF, type B natriuretic peptide, and type C natriuretic peptide, as well as truncated forms of natriuretic peptides containing as few as five amino acids from their ring structure (2–4). C receptors are more abundant than guanylyl cyclase receptors and are localized in tissues and cells that receive a large fraction of the cardiac output, including vascular endothelial and smooth muscle cells and the renal cortex (2). Blockade of C receptors by specific ligands decreases the metabolic clearance of ANF and consequently increases its plasma levels in mammals (3, 4). Thus, a major function of C receptors is to serve as a hormone buffer system to impede inappropriate alterations in plasma levels of natriuretic peptides (3). Although there is some controversy over whether C receptors may also function as signaling receptors, it has been well documented that they do not mediate the major known cardiovascular, adrenal, and renal effects of natriuretic peptides (2, 5).

The C receptor is a ~60-kDa protein that is constitutively present in the cell membrane as a homodimer of ~120 kDa (2). The receptor has a large NH2-terminal extracellular ligand binding domain, a single transmembrane domain, and a short COOH-terminal cytoplasmic tail of only 37 amino acids (6). A short cytoplasmic domain is a structural characteristic of all clearance and/or transport receptors whose structure is known, including low density lipoprotein, asialoglycoprotein, transferrin, cation-independent mannos-6-phosphate-IGF type II, and polymeric immunoglobulin receptors (7–13). C receptors have a single tyrosine in their cytoplasmic domain (Tyr508), an amino acid that has been shown to play a central role in clathrin-coated pit endocytosis of receptors (7–13). However, the flanking sequences of tyrosine in the C receptor do not bear any resemblance to the degenerate sequences that contribute to rapid internalization of other receptors (e.g. NPXY or YXXZ, where X is any amino acid, and Z is a large hydrophobic amino acid) (7). Moreover, there are no other recognized internalization motifs (e.g. dileucine) in the cytoplasmic domain of C receptors.

Our previous studies demonstrated that native C receptors in mammalian cells undergo rapid constitutive endocytosis that is not stimulated further by ligand binding. Endocytosed receptor-ligand complexes are then dissociated intracellularly, most probably in endosomes, with a subsequent hydrolysis of the ligand in lysosomes and an efficient recycling of the internalized receptors to the cell surface (14). In the present work we studied some of the molecular determinants of the clearance function of C receptors using cloned wild type and mutated human kidney C receptors transfected into Chinese hamster...
Endocytosis of Natriuretic Peptide Clearance Receptors

ovary (CHO) cells.

The results show that the internalization of C receptors via clathrin-coated pits depends on the integrity of their cytoplasmic domain. Within this domain, tyrosine contributes significantly to rapid endocytosis, albeit to a lesser degree than in other receptors in which it is flanked by conserved internalization motifs. Constitutive dimerization of C receptors, a property conferred by an unpaired cysteine residue in the extracellular domain, markedly enhances endocytosis, demonstrating that constitutive polymerization is an important determinant for rapid internalization of these receptors.

**EXPERIMENTAL PROCEDURES**

**Materials**—[35S]s-st-A TP for sequencing and [125I] for radiodiagnosis were obtained from Amersham Corp. [35S]Methionine-[35S]Cysteine mixture was obtained from DuPont NEN. All restriction enzymes and M13mp18 RF were purchased from New England Biolabs, Beverly, MA. Host strains of Escherichia coli (J11603, MC1061) and the DNA sequencing kit were obtained from U. S. Biochemical Corp. Rat ANF, 28 was purchased from Peninsula Laboratories Inc., Belmont, CA. Fetal and bovine calf serum were purchased from HyClone Laboratories, Logan UT. Unless otherwise indicated, all other chemicals, including culture media, enzymes, antibiotics, immunoochemicals, buffers, and reagents were purchased from Sigma.

**Mutagenesis**—The full-length recombinant human kidney C receptor was subcloned in the EcoRI cloning site of M13mp18 (15). Digestion with Smal permitted selection of the correctly oriented vector, which was then used for oligonucleotide-directed mutagenesis using either Zoller and Smith or Kunkel et al. methods (16, 17). Sense mutagenic oligonucleotides containing at least 7 bases flanking the codon to be modified were synthesized and annealed to the wild type receptor (CRWT), according to standard techniques (18). The sequence of amino acids of the CRWT region of interest for the present study, and the corresponding sequences of the mutated receptors are depicted in Fig. 1. The codons in the published nucleotide sequence of CRWT (19) were modified by a codon change protocol to determine the steady-state distribution of CRWT in the cell membrane and in the intracellular compartment (14).

**Ligand Binding and Cellular Distribution of Receptors—Equilibrium competition binding experiments to obtain the apparent dissociation constant (Kd) of receptor-ligand complexes and the apparent density of surface receptors (Bmax) were performed at 4°C, as published previously (14). Trypsinization-solubilization experiments in the absence of 10 mM NH4Cl were performed to determine the endocytic rates of CRWT in CHO cells and in the intracellular compartment (14).

**Culture of Wild Type and Recombinant CHO Cells—** Wild type and recombinant CHO-K1 cells were incubated in the presence of excess (0.5–1.0 mM) unlabeled ANF 28. Experiments were performed at the end of incubation time, the medium was removed for determination of precipitable and soluble radioactivity using the precipitation solution described above. Then, the monolayers were rapidly washed with ice-cold Hanks’ balanced salt solution and incubated with 1–2 ml of incubation medium in a shaking water bath at 37°C for several time intervals, up to 60 min. The incubation medium consisted of 1–2 ml of Dulbecco’s modified Eagle’s medium, 2 mg/ml bovine albumin and 0.4 mCi/well [35S]methionine-[35S]cysteine. After the incubation period, the monolayers were washed again with ice-cold Hanks’ balanced salt solution and incubated with 1 ml of 0.1% Triton X-100 in 2N NaOH at 1 h at room temperature to determine intracellular (acid-sensitive) radioactivity. The radioligand specifically bound to the cell surface remained nearly constant from 20 to 60 min of incubation (see Fig. 4). This allowed us to use a simplified steady-state assay to determine lysosomal hydrolysis and thereby to determine the endocytic rate of C receptors in the absence of 10 mM NH4Cl.
specific binding, internalization, and non-receptor-mediated metabolism of radioactivity; and
pied receptors internalized per min; mammalian cells. The apparent affinity (125I-ANF1–28 were
numbers in superscripts. The heavy lines indicate that the cytoplasmic tail was truncated at arginine 514 (CHOCR514) or
The underlined alanines in bold are point mutations replacing the correspondingly numbered amino acids in
CHO cells. The apparent density (Bapp) of surface receptors and their apparent ligand affinity (Kd)
in each of the recombinant cell lines, as determined by equilibrium competition binding experiments with 125I-ANF1–28 ("Experimental Procedures").
Measurements and Calculations—To assess intact 125I-ANF1–28 and labeled hydrolytic products, samples of medium were precipitated with an equilibrium of precipitation solution, as described above. The radioactivity in precipitated and soluble fractions of media, acid washes, and cell extracts was counted in a gamma scintillation counter. HPLC analysis of the nature of the soluble radioactivity in the medium revealed that it coeluted with [125I]monoiodotyrosine. Cells were counted with a hemocytometer. Net endocytic rates were determined by the formula ER = ([HIM + IC]/M) × 100/t, where ER is the net endocytic rate of surface receptor-ligand complexes expressed as percent of occupanted receptors internalized per min; HIM is the specific soluble radioactivity in medium; IC is the specific acid-insensitive (intracellular) radioactivity; M is the specific acid-sensitive (membrane-bound) radioactivity; and t is the time (in min) of incubation at 37°C. Non-specific binding, internalization, and non-receptor-mediated metabolism of 125I-ANF1–28 were < 10% of the corresponding total values and were subtracted from these values to calculate the net endocytic rate. Lysosomal hydrolysis was determined by the specific NH4Cl-sensitive soluble radioactivity in the medium. Quantitative results are expressed as mean ± S.E. Statistical analysis was performed using one-way analysis of variance followed by the Tukey-Kramer multiple comparison test among all groups. Differences were considered significant when p < 0.05.

RESULTS

Fig. 1 depicts the partial sequences of wild type and mutated C receptors, the apparent receptor-ligand (125I-ANF1–28) equilibrium dissociation constants (Kd), and the apparent surface densities (Bmax) of C receptors in the recombinant cells. The experiments revealed that specific binding of 125I-ANF1–28 to nontransfected CHO-K1 cells was negligible, a finding that was confirmed by immunocytochemistry (see Fig. 2) and immunoprecipitation (see Fig. 3) The recombinant cell clones used in the experiments were selected to have an apparent density (Bmax) in the range of those reported for native C receptors in mammalian cells. The apparent affinity (Kd) for ANF1–28 in CHOCRWT and in all cells expressing point-mutated receptors was < 0.1 nM, a value similar to that reported previously for native C receptors in mammalian cells (2). The apparent Kd of recombinant receptors with total deletion (CHOCR514) or large truncation (CHOCR514A) of the cytoplasmic domain was still low (< 1 nM) but significantly higher than that of the wild type and point-mutated C receptors (p < 0.01). C-ANF1–28 (a specific ligand of C receptors) displaced specifically bound 125I-ANF1–28 from CHOCRWT with a Ki similar to that reported previously for native C receptors (3) (data not shown). Trypsinization-solubilization experiments in recombinant CHO cell lines stably transfected into CHO cells. The lysate of nontransfected (CHO–) and recombinant (CHO–X-H) CHO cell clones fixed with formaldehyde were exposed to mouse monoclonal antibody against human kidney C receptors and visualized for optical microscopy immunofluorescence with anti-mouse IgG-fluorescein isothiocyanate conjugate as described under "Experimental Procedures." High intensity specific fluorescence is seen in all recombinant CHO cell lines but not in nontransfected CHO cells. Panel A, CHO– (nontransfected); panel B, CHOCRWT; panel C, CHOCR514; panel D, CHOCR514A; panel E, CHOCR5A80; panel F, CHOCR5A38; panel G, CHOCR5A80AS38; panel H, CHOCR473.
CHO cells showed that at equilibrium, 68.4 ± 6.9% or two-thirds of the total number receptors expressed in these cells were localized in the cell membrane (n = 5 wells in two independent experiments), a value similar to that found for native C receptors in bovine aortic vascular smooth muscle cells (14).

Fig. 2 shows the immunofluorescence signal in nontransfected CHO cells and in CHO cells transfected with wild type or mutated C receptors used in the present experiments. The results show that all recombinant cells, but not nontransfected CHO cells, displayed an intense fluorescence signal. No obvious qualitative differences were observed between the morphological expression of transfected wild type and mutated receptors in the recombinant cells at the resolution of the optical microscope.

Fig. 3 shows an SDS-PAGE autoradiogram of extracts from recombinant CHO cells immunoprecipitated with a specific monoclonal antibody against C receptors. In nontransfected cells, immunoprecipitated bands were very faint, if at all present, confirming that CHO-K1 cells do not express a significant density of native C receptors. As expected, under reducing and nonreducing conditions, the truncated forms of recombinant receptors (CHOCRWT−, and CHOCR514t) migrated at slightly lower molecular weights than CHOCRWT or the point mutated C receptors. Additional bands of unknown nature appeared in CHOCR514t under nonreducing conditions. They are probably an artifact, because under reducing conditions a single high intensity band was detected with this mutant. Under nonreducing conditions the recombinant receptors, except CHOCRA473, migrated as two bands, a higher density band of 100–120 kDa, and a lower density band of 50–60 kDa. CHOCRA473 migrated as a single band of 60 kDa. Under reducing conditions, the migration pattern of CHO- CRA473 remained unchanged, whereas in all other recombinant cells there was a virtual disappearance of the 100–120-kDa band and a prominent intensity of the 50–60-kDa band. Thus, all recombinant receptors, except CRA473, were present mainly as homodimers with a small proportion of monomers. This latter pattern is typical of native C receptors in several cell types (2). The finding that CRA473 was only present in monomeric form under nonreducing conditions confirms previous observations by other investigators showing that cysteine 473 is essential for C receptor dimerization (24, 25).

Fig. 4 depicts the results of a typical experiment on receptor-mediated internalization and hydrolysis of 125I-ANF1–28 in CHOCRWT cells. Panels A and B show the time course of specific binding of 125I-ANF1–28 to the cell membrane, the accumulation of intracellular radioactivity, and the appearance of 125I-ANF1–28 radioactive metabolites in the medium in control conditions (panel A) or in the presence of 10 mM NH4Cl (panel B). In control conditions, specific membrane-bound 125I-ANF1–28 remained nearly constant from 10 to 60 min, indicating rapid equilibration and lack of ligand-induced down-regulation of surface receptors during the course of incubation. Shortly after the start of the incubation, radioactivity accumulated in the intracellular compartment, reaching a peak by 10 min, and then maintaining near steady-state levels for the remainder of the experiment. In control conditions, there was a near linear increase in the appearance of labeled degradation products in the medium, and by 60 min the sum of degraded radioactivity in the medium and intracellular radioactivity far exceeds that of 125I-ANF1–28 bound to the cell surface, demonstrating that internalized recombinant receptors are replenished at the cell surface. The protein synthesis inhibitor cycloheximide did not alter this pattern (not shown), indicating that the replenishment of internalized receptors at the cell surface is due to recycling rather than de novo synthesis of receptors. Panel B shows that NH4Cl had practically no effect on the initial binding of radioligand to cell surface receptors or the initial (first 5–10 min) internalization rates of receptor-ligand complexes. At steady state, membrane-bound specific radioactivity in NH4Cl-treated monolayers was slightly but consistently higher than in control cells. The major effect of NH4Cl, however, was to block almost completely the appearance of labeled degradation products in the medium, confirming that this lysosomotropic weak base inhibits the lysosomal hydrolysis of internalized 125I-ANF1–28 (14). As a result, intracellular accumulation of radioactivity in NH4Cl-treated monolayers was approximately three times greater than in control cells by 60 min. The dynamics of recombinant wild type C receptor-ligand complexes in CHO cells shown in Fig. 4 is very similar to that determined previously in our laboratory for native C receptors in bovine aortic vascular smooth muscle cells (14).

In the experiment shown in Fig. 4, the calculated net endocytic rate of receptors-ligand complexes, which measures the difference between the rates of internalization (endocytosis) and return of undissociated receptor-ligand complexes to the cell surface (reendoctruciton) (14), was maximal at 5 min (9.6% of occupied receptors internalized per min), the first time of measurement, and then reached near steady-state levels between 10 and 60 min (−6%/min). Hereafter, all results on endocytic rates are expressed as steady-state net internalization rates (see "Discussion").
Endocytosis of Natriuretic Peptide Clearance Receptors

Recombination CHO cells stably transfected with wild type and cytoplasmic truncated forms of natriuretic peptide C receptors in control conditions or in the presence of hypertonic sucrose. Recombinant CHO cell monolayers were incubated in six-well plates at 37 °C in 2 ml of binding medium containing 0.3–0.5 mM NH4Cl. Experiments were also performed in control (Ctrl) conditions or in the presence of 0.44 M sucrose (HS). The times indicated on the abscissa of the upper graph, 0.1 ml of medium was removed to determine 125I-ANF1–28 hydrolytic products in medium, as described under “Experimental Procedures.” The endocytic rate of receptor-ligand complexes was calculated as described under “Experimental Procedures.”

**Fig. 5.** Receptor-mediated lysosomal hydrolysis of ANF, and net endocytic rate of receptor-radioligand complexes in recombinant CHO cells expressing wild type and cytoplasmic truncated forms of natriuretic peptide C receptors in control conditions or in the presence of hypertonic sucrose. Recombinant CHO cell monolayers were incubated in six-well plates at 37 °C in 2 ml of binding medium containing 0.3–0.5 mM NH4Cl. Experiments were also performed in control (Ctrl) conditions or in the presence of 0.44 M sucrose (HS). At the times indicated on the abscissa of the upper graph, 0.1 ml of medium was removed to determine 125I-ANF1–28 hydrolytic products in medium, as described under “Experimental Procedures.”

**Fig. 6.** Summarizes the values for receptor-mediated lysosomal hydrolysis of 125I-ANF1–28 (upper panel) and the net endocytic rate of receptor-ligand complexes (lower panel) in recombinant cells expressing point-mutated C receptors. Replacement of Phe538 (CHOCRA538) by Ala did not significantly affect lysosomal hydrolysis (220 ± 11% of specifically bound 125I-ANF1–28 by 60 min) or net endocytic rate (6.55 ± 0.92%/min) compared with CHOCRWT (p > 0.05). Replacement of Tyr508 (CHOCR508) or Cys473 (CHOCR473) by Ala significantly reduced lysosomal hydrolysis of 125I-ANF1–28 and net endocytic rates of receptor-radioligand complexes by approximately 40–50% compared with CHOCRWT or CHOCR508 (p < 0.001). However, net endocytic rates in CHOCR508 and CHOCR473 were still significantly greater than in cytoplasmic-truncated receptors or in sucrose-treated CHOCRWT (p > 0.01 versus CHOCRWT). In recombinant cells expressing the double mutant C receptor (CHOCR508A538), net endocytic rate and lysosomal hydrolysis of 125I-ANF1–28 were reduced to an extent similar to that in recombinant cells expressing the single Ala508 mutant (p < 0.01 versus CHOCRWT or CHOCR508; p > 0.05 versus CHOCR508).

The slopes of the regressions line defining the time course of the lysosomal hydrolysis of 125I-ANF1–28 of all point-mutated receptors, except CHOCR508 (Fig. 6, upper panel), were significantly smaller than that in CHOCRWT (p < 0.001). However, the slopes of lysosomal hydrolysis of all point-mutated receptors were significantly greater than those of the cytoplasmic truncated receptors (p < 0.001 versus CHOCRWT). Hypertonic sucrose did not affect ligand binding in CHOCR508, CHOCR508 or CHOCR508A538 but, for unknown reasons, caused a time-dependent dissociation of surface receptor-ligand complexes in CHOCR473. Thus, sucrose-
Figure 6. Receptor-mediated lysosomal hydrolysis of ANF, and net endocytic rate of receptor-rodilagand complexes in recombinant CHO cells stably transfected with point-mutated natriuretic peptide C receptors in control conditions or in the presence of hypertonic sucrose. See legend of Fig. 5 for description. Ctrl, control; HS, hypertonic sucrose; CRA538, CRA508, CRA508/538, and CRA473 are recombinant CHO cells transfected with C receptors in which alanine replaced phenylalanine 538, tyrosine 508, tyrosine 508, and phenylalanine 538 in the cytoplasmic domain, or cysteine 473 in the extracellular domain, respectively. Results are the mean ± S.E. of at least six wells in three separate experiments, except in the experiments with hypertonic sucrose (two wells). Hypertonic sucrose reduced lysosomal hydrolysis in all recombinant cell lines to negligible values (not shown). The net endocytic rate of CRA538 was not significantly different (p > 0.05) from that of CRWT in Fig. 5 and was significantly faster (+, p < 0.001) than that of all other point-mutated receptors.

Discussion

The dynamic characteristics of cloned wild type C receptors expressed in CHO cells are qualitatively and quantitatively very similar to those determined previously in our laboratory for native C receptors in cultured bovine aortic vascular smooth muscle cells (14). Recombinant wild type and native C receptors have similar ligand binding characteristics (Kd < 0.1 nM) and nearly equal net rates of endocytosis (5–7% of receptors internalized per min) and NH4Cl-sensitive (lysosomal) hydrolysis of ligand. There is no detectable difference between the cellular compartmentalization of recombinant CRWT in CHO cells and native C receptors in bovine aortic vascular smooth muscle cells (∼2/3 in the membrane and 1/3 in the intracellular compartment), and endocytosed native or recombinant wild type receptors undergo a highly efficient process of recycling to the cell membrane. These similarities indicate that ligand binding, receptor-ligand endocytosis, and cellular trafficking of internalized ligand and receptors are dependent on the molecular nature of C receptors rather than on the cell type in which they are expressed. Thus, it is likely that the present results on the molecular determinants of the clearance function of cloned C receptors can be fairly extrapolated to native C receptors in the mammalian organism.

Previous studies from our laboratory demonstrated that native C receptors are internalized constitutively with an endocytic rate similar to that of receptor-ligand complexes, a property that it also shares with other clearance and/or transport receptors (14). In the present study, hypertonic sucrose, which causes disassembly of clathrin and in this manner disrupts receptor-mediated endocytosis (13, 23), virtually abolished rapid endocytosis of C receptors. Thus, it is likely that constitutive internalization of C receptors, similar to other receptors, occurs via coated pits.

We chose to express our results as net endocytic rates of receptor-ligand complexes because in steady-state conditions this is likely to be the meaningful physiological parameter to assess the removal of ANF or other natriuretic peptides from the circulation. Moreover, it is not feasible to determine true unidirectional endocytic rates of receptors without preloading the ligand at 4°C and then warming the cells to 37°C to initiate the endocytic process. However, we have shown previously that temperature transition (4–37°C) per se may alter the dynamics of the constitutive internalization of C receptors (14). Under similar experimental conditions of steady-state and long term incubation, the net endocytic rate of recombinant CRWT receptor-ligand complexes (−6%/min) is comparable to that determined for asialoglycoprotein and cation-independent mannose 6 phosphate receptor-ligand complexes, and 30–50% lower than that of low density lipoprotein receptor and transferrin receptor-ligand complexes (7–10, 13). It is noteworthy that a net internalization rate of 5–7%/min for C receptor-ligand complexes is more than sufficient for an effective removal of natriuretic peptides from the circulation. Thus, the off rate of ANF from surface C receptors at 37°C is much slower than the rate of receptor internalization, allowing for a sufficient resident time for ligand internalization. Moreover, the high density of surface C receptors and the rapid cycling of internalized receptors to the cell surface contribute greatly to an efficient participation of the C receptor in the plasma clearance and homeostasis of natriuretic peptides in the intact organism (2, 4, 26).

The fundamental internalization signal of C receptors, similar to all other endocytosed receptors reported to date, resides in the cytoplasmic domain. Only complete deletion of cytoplasmic tail brings rapid endocytosis of C receptors to a virtual halt, i.e. the net internalization rate of the tailless receptor is approximately 10-fold lower than that of wild type receptors and not significantly different from that observed when endocytosis of wild type C receptors is blocked by hypertonic sucrose. A large deletion of the cytoplasmic domain (CHOCR514t) also markedly decreases the net endocytic rate of receptor-ligand complexes by ∼75%. This decrease is not due to the presence of a phenylalanine (Phe338) in the truncated region, as the net endocytic rate in the mutant CHOCR538 was not significantly different from that in CHOCRWT. Although it cannot be ruled out that there may be an internalization signal in the truncated region, it is more probable that the truncation of 28 of the 37 amino acids of the cytoplasmic domain disrupted the secondary structure of this domain and in this manner, its interaction with clathrin adaptor proteins.

In a strict sense, the C receptor does not contain in its cytoplasmic tail classic internalization motifs for coated pit internalization found in other receptors that undergo rapid endocytosis (e.g. NPXY, YXXZ, where X is any amino acid and Z is a large hydrophobic amino acid). However, C receptors...
have a cytoplasmic domain tyrosine (Tyr508), four amino acids removed from the putative single transmembrane domain. Replacement of this tyrosine by alanine decreases C receptor endocytosis by about 50%. This is a lower decrease than the 3–4-fold reduction reported for low density lipoprotein, transferrin, and asialoglycoprotein receptors when the tyrosine within the NPXY or YXXZ motifs was replaced by nonaromatic amino acids (7–9, 13). The residual endocytosis of receptor-ligand complexes in CHO-CRAS08 cells, as well as in all other recombinant cells, is almost completely blocked by hypertonic sucrose, indicating that the endocytosis of mutant receptors still occurs via clathrin-coated pits. Moreover, it is apparent that the mutations did not alter the routing of internalized radioligand to lysosomes or the recycling of dissociated receptors to the cell surface.

In view of the lack of an identifiable motif sequence for internalization of the C receptor, it is useful to compare the amino acid sequence of its cytoplasmic domain with that of a bona fide membrane resident protein that was mutated to contain a cytoplasmic tail tyrosine. An example of such a protein is the influenza virus hemagglutinin, which can be induced to internalize at a rate of 4–5%/min, a value very similar to that of wild type C receptors, by inserting a tyrosine in the cytoplasmic domain, five amino acids removed from the putative transmembrane domain (27). The added tyrosine that confers a rapid internalization signal to the influenza virus hemagglutinin is separated from the putative transmembrane domain by five amino acids (Asn-Gly-Ser-Leu-Gln), which have no homology with the three amino acids (Arg-Lys-Lys) that separate Tyr508 from the putative transmembrane domain of the C receptor. On the other hand, the COOH-terminal sequence flanking tyrosine, namely, Arg-Ile-Cys-Ile, for the influenza virus hemagglutinin, and Arg-Ile-Thr-Ile for the C receptor, share a high degree of homology. However, the sequence YRI does not seem to correspond to any of the postulated internalization motifs described to date, and therefore its biological meaning is unknown.

The present results, compared with those obtained in mutagenesis experiments with other receptors, further strengthen the notion that internalization motifs are degenerate, making it difficult to assign a generic amino acid sequence for rapid endocytosis on the basis of nature, size, hydrophobicity, or charge of the amino acids that flank the key aromatic amino acid tyrosine or, in some instances, phenylalanine (7–13). It has been postulated that the main importance of the tyrosine in the cytoplasmic domain of receptors is that this aromatic amino acid lies on a b-turn that confers the proper secondary structure for interaction with clathrin adaptor proteins (7). Recently, however, it has been shown by direct NMR analysis of angiotensin receptors that this may not be true for all membrane receptors (28). Indirect theoretical assessment of the secondary structure of the cytoplasmic domain of C receptors predicts that Tyr508 is part of a b-turn using Garnier et al. (29) and a b-sheet using Chou and Fasman (30) algorithms. In both models, however, replacement of Tyr508 by Ala predicts the introduction of an a-helix in place of a b-turn or a b-sheet in the cytoplasmic domain of C receptors. In view of the low intrinsic accuracy of existing models to predict secondary structure of proteins, direct structural analysis of C receptors is needed to elucidate the role of Tyr508 in conferring a structural motif for the internalization of C receptors.

Native or recombinant C receptors are mostly present at the cell surface as homodimers, with a small proportion of monomers (Fig. 3). The present results demonstrate that constitutive dimerization plays a significant role in rapid endocytosis, as the dimer forms of C receptors internalize at approximately twice the rate of monomer forms in which Cys473 was replaced by Ala. The duplex cytoplasmic domain in the dimer forms may interact more strongly with adaptor proteins in clathrin-coated pit, leading to a faster endocytic rate, or alternatively, it is possible that dimer forms are more easily trapped in coated pits because of a lower lateral mobility in the plane of the membrane. The present results do not permit us to distinguish between these two possibilities. Whatever the case, the constitutive predominance of homomers of C receptors is likely to increase the efficiency of receptor-mediated endocytosis of ligand by approximately 4-fold because dimers are able to bind the double of ligand molecules, and internalize at twice the rate of monomers. Thus, constitutive dimerization is particularly important for the clearance function of C receptors. To our knowledge this is the first demonstration that constitutive polymerization markedly enhances receptor endocytosis. It remains to be investigated whether this is also the case for other clearance and/or transport receptors.

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