VXFD in the Cytoplasmic Domain of Macrophage Scavenger Receptors Mediates Their Efficient Internalization and Cell-Surface Expression

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We determined the endocytosis motif sequence of macrophage scavenger receptors by measuring the internalization of the ligand-bound cell-surface scavenger receptors at the early stage. Mutation experiments have indicated that the 28 amino acids at the N-terminal cytoplasmic domain are important for both efficient internalization and cell-surface expression of the receptor. The substitution of alanine for Val21, Phe23 or Asp24 reduced both the efficiency of internalization and the cell-surface expression of the receptors; in addition, that for Val28 reduced only the internalization efficiency and that for Lys22 reduced only the cell-surface expression of the receptors. Western blot experiments showed that the total levels of expression of the mutant receptors in the cells were approximately the same and all the mutants were identified in the membrane fraction. This suggests that the Val21, Lys22, Phe23 and Asp24 mutants are localized in membranes other than the cell-surface membrane by missorting. All our results indicate that VXFD is the motif sequence essential for not only the coated-pit-mediated endocytosis of macrophage scavenger receptors but also for the trafficking of the receptors to the cell surface.

Key words macrophage scavenger receptor; endocytosis; cell-surface expression

Macrophage scavenger receptors bind and take up modified low density lipoproteins (LDL),¹⁾ which results in the deposition of cholesterol in macrophages.²⁾ Excessive uptake of cholesterol converts the macrophages into foam cells.³⁾ This event is a typical phenomenon observed in the initial stages of atherogenesis in arterial walls.⁴⁾ In a previous study, we had shown that modified LDLs, such as acetylated LDLs (AcLDLs), were internalized by macrophage scavenger receptors *via* receptor-mediated endocytosis.^{1,5)} Macrophage scavenger receptors spontaneously gather in coated pits on the cell surface as in the case of LDL receptors, while epidermal growth factor (EGF) receptors require ligand binding for clustering at the coated pits.^{5,6)} The amino acid sequences essential for efficient coated-pit-mediated internalization have been determined for several receptors, *e.g.*, NPXY for the LDL receptor,^{7,8)} YXRF for the transferrin receptor,^{9,10)} YKYSKV for the cation-independent mannose 6-phosphate/insulin-like growth factor-II receptor,¹¹⁾ FPHLAF and YRGV for the cation-dependent mannose 6-phosphate receptor,¹²⁾ YRAL and QQGFF for the epidermal growth factor receptor.^{13,14)} It is predicted that most of these motif sequences take the form of a turn structure.⁶⁾ In fact, the solution structure of the motif sequences essential for internalization of the insulin receptor and LDL receptor has been determined to be a tight turn structure by NMR.^{15,16)} There is an interesting report of a synthetic sequence of NNAYF, similar to the endocytosis motif sequence for the LDL receptor and predicted to have a tight turn structure, being effectively able to replace the motif sequence of the epidermal growth factor receptor.¹⁴⁾ These findings indicate the importance of the turn structure for efficient endocytosis. In the case of macrophage scavenger receptors, no such known internalization sequences have been identified in the cytoplasmic domain. This suggests that macrophage scavenger receptors must have another internalization signal sequence. In this report, we describe our attempts to determine the internalization motif se-

quence of macrophage scavenger receptors. We constructed a variety of mutant receptors and investigated their internalization efficiencies. We found that a novel motif sequence, which is predicted to form a helix structure, was responsible for both the efficient endocytosis and cell-surface transport of macrophage scavenger receptors.

MATERIALS AND METHODS

Construction of Mutant Receptors For constructing the deletion-mutant genes, we used the PCR method to produce the deleted DNA fragments. Forward oligonucleotide primers coded for the N-terminal 8 amino acids of the cytoplasmic domain along with extra methionine and alanine at the N-terminus, and having a *Bam*HI site at the 5' end, and reverse primers which were hybridized with spacer domain DNA containing the *Bsp*EI site, were synthesized and used for the PCR with pXSR7 as the template. The PCR products were directly subcloned into the pCRII vector. The nucleotide sequences of these PCR products were determined by the dideoxy sequencing method. The plasmids were then digested with *Bam*HI and *Bsp*EI, and the inserts were isolated. pXSR7 was digested with *Bam*HI and *Bsp*EI to remove intact the cytoplasmic and transmembrane regions and a part of the spacer domain region. This fragment was then ligated to the subcloned PCR products.

Point mutations were induced using the PCR primers. For this purpose, we generated new restriction sites, *Bss*HII or *Bst*EII, proximal to the target site of the mutations, without changing the amino acid sequence. Two mutated PCR products (5'- and 3'-frgs) were ligated at this restriction site. The 5'-frgs were produced by PCR using a forward 5'-primer (5'-CAGATATACGCGTTGACA-3') containing the *Sna*BI site ~400 bp upstream of the translation start site, as well as the reverse primers for the mutations, with pXSR7 as the template. The 3'-frgs were produced by the same method as that

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for the 5'-frgs using the forward primers for the mutations and the reverse primer (5'-AATCCTTTTCTGTGGATA-3') containing the *PacI* site ~80 bp downstream of the translation termination codon. These PCR products were subcloned into the pCRII vector, and their nucleotide sequences were determined by the dideoxy sequencing method. Then, the 5'-frg was cut out with *SnaBI* and either *BssHII* or *BstEII*, and the 3'-frg was cut out with either *BssHII* or *BstEII* and *PacI*. The vector was prepared by the digestion of pXSR7 with *SnaBI* and *PacI*. The vector and the 5'- and 3'-frgs were ligated to yield plasmids for the expression of the point mutant receptors.

Internalization Index and Cell-Surface Expression

The plasmids with deletion or point mutations were introduced into COS-7 cells by the DEAE-dextran method.¹⁷⁾ Two days after transfection, the cells were used for the assay. After the cells were chilled to 4 °C for 30 min, ice-cold ligand-DMEM medium (10% fetal bovine serum (FBS), 5 µg/ml ¹²⁵I-acetyl-LDL) was added, and the cells were allowed to stand on ice for 1.5 h. Then, the labeled solution was removed and the warmed (37 °C) DMEM medium (10% FBS, 5 µg/ml nonlabeled acetyl-LDL) was added and the cells were incubated again for exactly 5 min at 37 °C. Immediately thereafter, the medium was replaced with ice-cold buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 2 mg/ml bovine serum albumin (BSA)). The cells were washed three times with this buffer at 4 °C and once with the other cold buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl). The surface bound ligands were removed by incubation with the cold buffer (10 mM HEPES NaOH (pH 7.4), 50 mM NaCl, 4 mg/ml dextran sulfate) on a rotary shaker at 4 °C for 1 h. The radioactivity in the recovered solution was counted. Then, 0.1 M NaOH was added to the cells and the counts in the solubilized cells were measured.

Western Blot Analysis HA1-tagged scavenger receptors and 12CA5 antibody (Boehringer Mannheim) were used for the Western blot analysis. pXSR7 was digested with *BcgI* to yield a positive control vector. By this treatment, the C-terminal 13 amino acids of the receptor were removed. This region is unrelated to the binding and uptake of ligands.¹⁾ The synthesized 37-bp fragment, coded for the HA1-tag (YPYDVPDYAS)¹⁸⁾ and stop codon, was ligated to the control vector. For the preparation of plasmids for HA1-tagged mutant receptors, the mutant plasmids were digested with *XbaI* and the resultant short fragment, coded for the C-terminal region of the receptor, was replaced with the fragment obtained by *XbaI* digestion of the plasmid for the tagged control receptor. COS-7 cells were transfected with these plasmids. Two days after transfection, 2×10⁴ cells were used for Western blot analysis. Receptor proteins on the nitrocellulose filter were detected using the 12CA5 antibody and the ECL Western blot analysis system (Amersham).

Cell Fractionation The fractionation of cells was based on the method of Sato *et al.*¹⁹⁾ and is described in Fig. 4. All operations were carried out at 4 °C. Cells were suspended in five equal volumes of HEPES buffer (10 mM HEPES-KOH at pH 7.6, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM EDTA, 1 mM PMSF, 20 µM leupeptin), disrupted by ten passages through a 21-gauge needle, and centrifuged at 100000×g for 1 h. The membrane pellet was resuspended in an equal volume of either 0.1 M sodium carbonate at pH 11,

or HEPES buffer. After incubation for 15 min at room temperature, the mixtures were centrifuged at 100000×g for 1 h.

RESULTS

There is no known motif sequence for efficient endocytosis in the cytoplasmic domain of macrophage scavenger receptors. In order to determine the region essential for efficient internalization, we constructed deletion-mutant genes of the cytoplasmic domain of the receptor and examined the internalization efficiencies of the expressed mutants in COS-7 cells. In the case of macrophage scavenger receptors, the cytoplasmic domain consists of an N-terminal end of the receptor. Thus, we expected that deletion of the N-terminal end might influence the cell-surface expression of the receptor. In order to accurately observe the internalization efficiency not affected by the amount of cell-surface expression, we measured the ratio of the amount of the internalized ligand following one-cycle endocytosis to the total amount of ligand bound to the receptor before endocytosis. The mutant-receptor-expressing COS-7 cells were chilled to 0 °C and ¹²⁵I-AcLDL was added to allow it to bind to the receptor. After removing the ligands that remained unbound to the receptor, the receptor-bound ligands were internalized by incubation at 37 °C for 5 min. We measured the radioactive counts of the internalized (A) and non-internalized ligands (B) at this time. Here, A plus B indicates the receptor-bound ligands before endocytosis. We therefore estimated the values of A/(A+B) as the internalization index. Because this value shows the ratio of the ligands internalized *via* binding to the receptor at the very early stage of endocytosis, this value is not affected by the amount of cell-surface expressed receptors. As shown in Fig. 1, we constructed the mutant receptor genes, then COS-7 cells were transfected with these genes. We measured the internalization efficiencies of these deletion mutants and the ligand binding of the mutants on the cell surface in one cycle endocytosis. Because the extracellular domain of scavenger receptors also has independent ligand-binding capability, we considered that the amino acid sequence of this domain would not affect the ligand binding. Actually, we measured the amounts of cell-surface expressed HA1-tagged receptors using the antibody, and found that the amounts of receptors correlated well with the values of the ligand binding. We therefore considered that the amount of ligand binding reflects the level of cell-surface expression. The results shown in Fig. 1 indicate that Δ(3-17) did not affect the internalization efficiency. However, Δ(3-19) resulted in a small decrease in internalization efficiency and Δ(3-27) resulted in a significant decrease. On the other hand, Δ(3-17) had no effect on the cell-surface expression, while both Δ(3-19) and Δ(3-27) decreased the level of expression on the cell surface.

The results of the deletion mutation experiments suggest that the region between amino acids 18 and 27 from the N-terminus includes the sequence crucial for efficient endocytosis. To precisely determine this motif sequence, we constructed a series of alanine-scan mutant genes in this region. These mutants were expressed in COS-7 cells and the same assays as those for the deletion mutants were performed. The results showed that point mutations V21A, F23A, D24A and V28A reduced the internalization efficiencies, as shown in Fig. 2.

Fig. 1. Internalization Index and Cell-Surface Expression of Cytoplasmic Domain Deletion Mutants of the Macrophage Scavenger Receptor

(A) Amino acid sequences of the native receptor and deletion mutants. The gray bar indicates the same amino acid sequence as the native receptor. (B) Internalization index and cell-surface expression of the native (pXSR7) and the deletion mutant receptor (p $\Delta(3-11)$ —p $\Delta(3-38)$). pcDNA1 indicates a plasmid without the receptor gene (negative control). In the case of cell-surface expression, values relative to the native receptor (pXSR7) are indicated.

Fig. 2. Internalization Index and Cell-Surface Expression of Cytoplasmic Domain Point Mutants of the Macrophage Scavenger Receptor

(A) Amino acid sequences of native receptor and point mutants. The gray bar indicates the same amino acid sequence as the native receptor. (B) Internalization index and cell-surface expression of the native (pXSR7) and the point mutant receptor (pS16A—pT29A). pcDNA1 indicates a plasmid without the receptor gene (negative control). In the case of cell-surface expression, values relative to the native receptor (pXSR7) are indicated.

In regard to cell-surface expression, we detected a remarkable decrease in expression in the case of V21A, K22A, F23A and D24A. In order to clarify whether or not this decrease is due to a decrease in protein synthesis, we carried out Western blot analysis for mutant receptors. The results showed that the expression levels of the mutant receptors were almost the same as that of the natural receptor, as shown in Fig. 3, and that all these mutant receptors existed as membrane proteins, as shown in Fig. 4. We therefore consider that the decrease in cell-surface expression of these mutants is due to a deficiency in the transport of protein from ER to the cell surface.

Altogether, our results strongly suggest that Val21, Phe23 and Asp24 are responsible for both the efficient internalization and cell-surface expression of macrophage scavenger receptors. In addition, Val28 is important for efficient internalization, and Lys22, for cell-surface expression.

DISCUSSION

For measuring the internalization index, we measured the

Fig. 3. Western Blot Analysis of the Expression of HA1-tagged Mutants and Native Receptor in Transfected COS-7 Cells

Square brackets indicate the positions of the receptor proteins. Whole cell extracts of 2×10^4 COS-7 cells transfected with plasmids were subjected to 10% SDS-PAGE followed by Western blot analysis. Endogenous actin was also detected with an anti-actin polyclonal antibody (IgG fraction) (Biomedical Technologies, Inc.) as the internal control.

Fig. 4. Subcellular Distribution of the Native and Cytoplasmic Domain Mutant Receptors in Fractions of Transfected COS-7 Cells

(A) Procedure of subcellular fractionation. (B) Western blot analysis of the fractionated protein. Arrows indicate the positions of the receptor proteins. The supernatants (S-1, S-2, S-3) and solubilized membrane pellets (P-2, P-3) and the solubilized whole cell extracts from 2×10^4 cells were subjected to 10% SDS-PAGE followed by Western blot analysis.

efficiency in one cycle of endocytosis. The degradation assay is not suitable for measuring the endocytosis efficiency because it includes both uptake and recycling efficiencies. In our protocol, the variation in cell-surface expression levels of the mutant receptors does not pose any serious problem. In particular, because the cytoplasmic domain of the receptor consists of its N-terminus, we expected a variation in the level of cell-surface expression. Although the differences in the internalization index were small because we measured one cycle of endocytosis, we could estimate precisely the internalization efficiencies of the mutant receptors.

Macrophage scavenger receptors are not known to have any internalization motif sequences in their cytoplasmic domain. It has been reported that one of the commonly adopted structures of the internalization motif sequences is a certain turn structure.^{6,15,16} This turn structure motif could also function on other receptor molecules.²⁰ In the case of scavenger receptors, we could detect two sites having a turn structure

(the Asp13—Glu19 and Asn38—Thr41 regions) by secondary structure prediction programs. Initially, we expected that either one of these two might be a crucial motif sequence for internalization of the scavenger receptors. However, the results of our deletion and point mutation experiments revealed that neither sequence was essential, and that the Val21—Val28 sequence was responsible for the efficient internalization of the receptor. Here, this sequence was estimated to adopt a helix structure, and not a turn structure, by secondary structure prediction. Only a couple of examples are known in which the internalization motif sequence is thought to adopt a helix structure, namely, CD4 and the angiotensin II receptor.^{21,22} In both cases, the formation of an amphiphilic helix structure was noted to be possible only when the amino acids were arranged on a helix. They did not directly prove the helix structure. Similar hydrophobic polarity on the helix could be observed in the motif sequence of the scavenger receptors. In order to determine whether the internalization motif sequence of the scavenger receptors investigated in this study adopts a helix structure, we constructed an 18 amino-acid-peptide including this motif sequence and measured its CD spectrum. We found that this peptide did not adopt a helix, but a random coiled structure (data not shown). This 18 amino-acid-peptide might not be sufficiently long to allow the formation of a definite structure. Or, a trimer assembly may be necessary for adopting an ordered structure.

We are now further investigating the structure and function of the cytoplasmic domain of macrophage scavenger receptors.

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