The calcium-independent receptor of α-latrotoxin (CIRL), a neuronal cell surface receptor implicated in the regulation of exocytosis, is a natural chimera of the cell adhesion protein and the G protein-coupled receptor (GPCR). In contrast with canonic GPCRs, CIRL consists of two heterologous non-covalently bound subunits, p120 and p85, due to endogenous proteolytic processing of the receptor precursor in the endoplasmic reticulum. Extracellularly oriented p120 contains hydrophilic cell adhesion domains, whereas p85 resembles a generic GPCR. We determined that the site of the CIRL cleavage is located within a juxtamembrane Cys- and Trp-rich domain of the N-terminal extracellular region of CIRL. Mutations in this domain make CIRL resistant to the cleavage and impair its trafficking. Therefore, we have named it GPS for G protein-coupled receptor proteolysis site. The GPS motif is found in homologous adhesion GPCRs and thus defines a novel receptor family. We postulate that the proteolytic processing and two-subunit structure is a common characteristic feature in the family of GPS-containing adhesion GPCRs.

Cell adhesion receptors provide physical links between cell plasma membranes and the extracellular matrix. These receptors have large extracellular domains that contain characteristic structural modules that are directly involved in cell-to-cell and cell-to-matrix interaction. They can also function as signaling receptors, providing the cell with critical information required for proper tissue growth and development. The signaling function of cell adhesion receptors has been primarily restricted to epididymis, thus suggesting its function in sperm maturation (11). Most of the other adhesion GPCRs were discovered by gene sequencing and have not been thoroughly characterized either functionally or biochemically. Among the adhesion GPCRs, only CD97 has a known binding partner, the membrane protein CD55 (or decay accelerating factor; DAF) (12). All other receptors remain orphan, i.e. their endogenous agonists and antagonists are not known yet.

In our earlier studies (7) of CIRL we made the unexpected finding that this receptor consists of two non-covalently bound fragments named p120 and p85. N-terminal amino acid sequencing suggested that the cleavage occurred between residues Leu837 and Thr838 in the extracellular region of CIRL close to the first transmembrane segment. Extracellularly oriented, hydrophilic p120 has structural features typical of a cell adhesion molecule, whereas p85 resembles a generic GPCR. Both subunits are transcribed from one gene, suggesting that proteolytic processing of the receptor precursor must occur. This finding raised questions as to whether this cleavage is a unique characteristic of CIRL and whether it may have a role in receptor regulation, as in the case of thrombin receptors (13).

To address these questions, we confirmed the site of the CIRL cleavage by mass spectrometry and determined the subcellular localization of this processing. Our data suggest that the two-subunit structure of CIRL is a result of the constitutive proteolytic processing of a novel type. The cleavage of CIRL occurs intracellularly very early in the biosynthetic pathway and is important for proper surface expression of the receptor. The site of the cleavage includes a cysteine- and tryptophan-rich motif, which is conserved in chimeric adhesion GPCRs. We

By this mechanism, cells should be able to produce fast and sensitive responses to physical contacts with the extracellular environment.

Putative cell adhesion GPCRs are linked to different cellular functions in a variety of tissues. CD97 and EMR1 (F4/80 antigen) are involved in leukocyte activation (3–6). The calcium-independent receptor of α-latrotoxin (CIRL), a neuronal target of a presynaptic neurotoxin, has been implicated in the regulation of secretion (7, 8). BAI, a p53-inducible protein, is an inhibitor of angiogenesis (9). The Drosophila receptor Flamingo has a role in establishing planar cell polarity, as pointed out by genetic studies (10). Expression of the HE6 receptor is restricted to epididymis, thus suggesting its function in sperm maturation (11). Most of the other adhesion GPCRs were discovered by gene sequencing and have not been thoroughly characterized either functionally or biochemically. Among the adhesion GPCRs, only CD97 has a known binding partner, the membrane protein CD55 (or decay accelerating factor; DAF) (12). All other receptors remain orphan, i.e. their endogenous agonists and antagonists are not known yet.
Proteolytic Processing of Adhesion GPCR

EXPERIMENTAL PROCEDURES

Miscellaneous Procedures— α-Latrotoxin was purified from lyophilized black widow spider glands and radioactively labeled with 32P by chloramine T procedure. α-Latrotoxin binding assays were performed as described (7, 14). The toxin was immobilized on BrCN-Sepharose as described (15). CIRL-1 encoding plasmid pCDR7 was obtained by cloning the original full-length CIRL-1 cDNA fragment into pcDNA3.1. The toxin was immobilized on BrCN-Sepharose as described (16). The toxin was immobilized on BrCN-Sepharose as described (17).

The CIRL-1/neurexin-Ia chimera was obtained by replacing the Myc/His6 tag of pSTR7-2 with the transmembrane and C-terminal intracellular regions of neurexin Ia. A HindIII-digested PCR fragment (with oligonucleotides 1 and 2 and pCDR7 as a template) was ligated with an AgeI/HindIII-digested PCR fragment (with oligonucleotides 3 and 4 and pcVM-L2 as a template). The ligation product was digested with AgeI/XbaI, isolated by DNA agarose electrophoresis, and ligated with the 7247-bp fragment of AgeI/XbaI-digested pSTR7-2. The resulting plasmid pSTR7-Nx10-CT-2 was confirmed by sequencing.

Antibodies— Three sets of polyclonal antibodies were used. The first set was directed against the C-terminal Myc/His6-tagged extracellular CIRL-1 domain. The second set was directed against the Myc/His6 tag of pSTR7-2. The third set was directed against the C-terminal Myc/His6-tagged extracellular CIRL-1 domain.

Peptides— Sample Preparation for MALDI-TOF Mass Spectrometry— Peptides were concentrated and contaminants removed by micro reverse phase chromatography on C18 silica resin (600-nl bed volume) pipette tip columns (Millipore C18 ZipTip™). Samples in 0.1% trifluoroacetic acid were applied, washed with 0.1% trifluoroacetic acid, and eluted in 1–2 μl of 90% acetonitrile and 0.1% trifluoroacetic acid. Crystals were formed using the dried droplet method by allowing mixtures of 0.5 μl of the sample and 0.5 μl of the matrix solution consisting of 10 mg/ml sinapinic acid in 0.1% trifluoroacetic acid and 40% acetonitrile to dry at room temperature.

MALDI-TOF Mass Spectrometry— Positive ion mass spectra were acquired using a Micromass ToSpec-2E MALDI-TOF mass spectrometer with time lag focusing using standard instrument settings. Data were acquired and processed using manufacturer-supplied MassLynx software.

Single Residue Mutants of CIRL-1—The point mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol by replication of both parental plasmid strands without displacing the mutant oligonucleotide primers. Three pairs of oligonucleotides were used to introduce mutations by replacing just one nucleotide in the parent DNA sequence. A set of oligonucleotides 5 and 6 (see the list below) was used to replace Trp717 with Ser, oligos 7 and 8 were used to replace Cys834 with Trp, and oligos 9 and 10 were used to replace Trp815 with Pro. The 1420-bp fragment of pCDR7 digested with SfiI/KpnI was cloned into bScript and used as a template to generate mutants. The three mutant plasmids were digested with HpaII/BspEI and the 1189-bp fragments with mutations were used to replace the wild-type fragment in pCDR7. The final plasmids pCDR7-C834/4, pCDR7-T815/P, and pCDR7- W717/S were expressed in the presence of the bacterial protease.

Immunofluorescence Analysis of Transfected HEK-293 Cells— On day 1, transient transfection was performed with 1 μg of plasmid per well. 48 h after transfection, culture media were aspirated, and cells were washed twice with Dulbecco’s phosphate buffered saline (DPBS, Invitrogen) and fixed in 4% freshly prepared formaldehyde fixative in DPBS at room temperature for 30 min. After fixation, the cells were rinsed twice with DPBS and, if necessary, permeabilized with 0.3% Triton X-100 and 1% goat serum in DPBS at room temperature for 30 min. After permeabilization, the slides were rinsed once with DPBS and incubated in a blocking solution (5% goat serum in DPBS) at room temperature for 1 h. The slides were further incubated with primary antibodies at room temperature for 45 min and gently washed without agitation three times in the blocking solution and three times in DPBS for 10 min each wash. Secondary antibodies were diluted in the blocking solution and applied to the slides for 30 min without agitation. The slides were washed in three changes of blocking solution and DPBS for 10 min each wash. After washing, the slides were mounted with Shur-Mount mounting media (Triangle Biomedical Sciences) and stored in the dark.

Pulse and Chase Labeling Experiments— COS cells in 100-mm dishes were transfected with 150 μg NaCl. On the next day, washed twice with 15 μl of phosphate-buffered saline and switched to 3 ml of cys- and methionine-free Dulbecco’s modified Eagle’s medium (ICN Pharmaceuticals) containing penicillin, streptomycin, 25 mM HEPES (pH 7.2), and 10% fetal calf serum. After incubation for 30 min at 37 °C, 0.7 μCi (80 μl of Tran^35 Label reagent (ICN Pharmaceuticals) was added per 100-mm Petri dish. The cells were incubated for another 30 min at 37 °C (pulse), and the medium was replaced with regular Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum; incubation continued for the indicated time (chase). After the chase period, the medium was moved, and the cells were lysed with 1.4 ml of cold 20 mM Tris-HCl, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride buffer, pH 8.0. The adsorbed proteins were eluted with the SDS sample buffer and further analyzed by Western blotting with either anti-p120 or anti-85 antibodies.

Purification of Soluble Myc-tagged p120— COS cells were transfected with pSTR7-2 and incubated after transfection in a serum-free medium. On day 3, the medium was harvested and clarified by centrifugation and concentrated by ultrafiltration with an Amicon P-10 filter to a final volume of 5 ml. The concentrate was centrifuged at 40,000 × g for 30 min and added to 100 μl of α-latrotoxin-agarose. After an overnight incubation at 4 °C, α-latrotoxin-agarose was collected by brief centrifugation and resuspended in 50 ml of Tris-HCl, 150 mM NaCl, 2 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride buffer, pH 8.0. The adsorbed proteins were eluted with 200 μl of 3 M MgCl2, 50 mM Tris-HCl buffer, pH 8.

Purification of p120/neurexin-Ia Chimera— COS cells were transfected with pSTR7-Nx10-CT-2. On day 3, the cells were harvested, resuspended in the buffer containing 50 mM Tris-HCl, 150 mM NaCl, and 2 mM EDTA, pH 8, and extracted with 2% Triton X-100. The extract was centrifuged at 100,000 × g for 1 h and further mixed with 100 μl of α-latrotoxin-agarose. After an overnight incubation at 4 °C, α-latrotoxin-agarose was collected by brief centrifugation and washed three times with 15 ml of ice-cold 50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, and 0.1% Triton X-100 buffer, pH 8. The adsorbed protein was eluted with 200 μl of 3 M MgCl2, 50 mM Tris-HCl buffer, pH 8.

Sample Preparation for MALDI-TOF Mass Spectrometry— Peptides were concentrated and contaminants removed by micro reverse phase chromatography on C18 silica resin (600-nl bed volume) pipette tip columns (Millipore C18 ZipTip™). Samples in 0.1% trifluoroacetic acid were applied, washed with 0.1% trifluoroacetic acid, and eluted in 1–2 μl of 90% acetonitrile and 0.1% trifluoroacetic acid. Crystals were formed using the dried droplet method by allowing mixtures of 0.5 μl of the sample and 0.5 μl of the matrix solution consisting of 10 mg/ml sinapinic acid in 0.1% trifluoroacetic acid and 40% acetonitrile to dry at room temperature.

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FIG. 1. Localization of the CIRL cleavage site. A, domain structure of CIRL and chimeras of its extracellular region with neurexin C-terminal domain or Myc epitope. B, proteolytic processing of p120/Myc in COS cells. The transfected cells were analyzed by Western blotting with anti-p120 or anti-Myc antibodies either directly (Cell) or after Triton X-100 extraction and purification on α-latrotoxin-agarose (Extr./LTX). The secreted protein was also purified from the conditioned medium and analyzed in parallel (CM/LTX). C, mass spectrometry analysis of the p120/myc cleavage product. The conditioned medium of COS cells transfected with p120/Myc was purified by α-latrotoxin-agarose chromatography and analyzed by MALDI as described under “Experimental Procedures.” D, proteolytic processing of the p120/neurexin chimera in COS cells. The transfected cells were lysed in the SDS sample buffer and analyzed by Western blotting with anti-p120 and anti-neurexin antibodies. The
RESULTS

In our original experiments, CIRL cDNA was cloned on the basis of partial amino acid sequences of p120, a glycoprotein purified from solubilized brain membranes by chromatography on an immobilized \( \text{H}11032 \)-latrotoxin column (7). The full-length CIRL cDNA encodes a protein of 1471 amino acid residues, which was more than expected, especially considering that at least 15% of the p120 mass can be accounted for by carbohydrate content. Also, an antibody against the C-terminal sequence of the cloned protein that worked very well in the immunoprecipitation of CIRL failed to stain p120 on Western blots and instead decorated a fuzzy band of \( \text{H}11011 \) 85 kDa. To explain these data, we postulated that CIRL consisted of two non-covalently bound heterologous subunits, p120 and p85, derived from endogenous posttranslational proteolytic processing of the full-length precursor protein (Fig. 1A, left).

To confirm the cleavage site location, we utilized high resolution mass spectrometry of the proteolysis products. Because p85 is too large for accurate detection, two CIRL mutants were prepared (see “Experimental Procedures”) that would have relatively small C-terminal fragments after the cleavage. The first construct encoded the extracellular domain of CIRL tagged with Myc-epitope at the C terminus (Fig. 1A, center). It was expected to produce a soluble secreted protein. The second construct was a fusion protein of CIRL extracellular domain with 75 amino acid residues of the single transmembrane domain and a cytoplasmic tail of neurexin I. This plasmid would express a membrane complex essentially similar to CIRL but with a substantially smaller “p85” subunit (Fig. 1A, right).

When transfected into COS cells, both constructs expressed well, and the protein products were cleaved as expected. All secreted p120/Myc was cleaved as revealed by Western blotting with anti-p120 and anti-Myc antibodies, whereas at least a significant portion of the intracellular protein was not processed, perhaps due to saturation of the processing protease (Fig. 1B).
Western blotting with anti-neurexin antibodies detected a small fragment equivalent to the p85 subunit of CIRL (Fig. 1D, gray arrow). The size of the p120 fragment of the cleaved chimeras was indistinguishable from p120 of wild-type CIRL (data not shown).

The mutant proteins were purified by affinity chromatography on α-latrotoxin-agarose and further analyzed by mass spectrometry. The highest peak seen in the spectrum of the soluble mutant had a mass of 3758.7 (M + H⁺) that matched very well with the calculated average mass (3759.1 Da, M + H⁺) of the peptide TNFAVLMASRGPEQKLISEEDLNSAVDH-HHHHH (Fig. 1C). This result suggested that the cleavage site localized N-terminally from Thr838, 19 residues from the first transmembrane region of CIRL. When the membrane-bound CIRL-neurexin chimera was purified and analyzed in the same manner, the major peak (10,752.9 Da) was noted in the region...
corresponding to the anticipated cleavage product starting at the Thr<sup>R38</sup> fragment (Fig. 1A). The broad peak at this m/z could be explained by partial protein (i.e. methionine) oxidation as a result of purification in the presence of the Triton X-100 that was included to solubilize the mutant protein. The data obtained by mass spectrometry of CIRL mutants were in good agreement with the previously described detection of the sequence that started from Thr<sup>R38</sup> by direct N-terminal sequencing of purified CIRL. We therefore concluded that the site of CIRL proteolytic processing that results in the complex of p120 and p85 lies between the residues Leu<sup>R37</sup> and Thr<sup>R38</sup> of CIRL.

Most of the described cases of receptor proteolysis involve furin-like proteases that cleave at basic residues. This was obviously different with CIRL, so we made an attempt to identify a potential consensus for the cleavage by a non-furin yet quite specific protease. When we used the CIRL cleavage site sequence CACSHL | TNFAVL to do a BLAST search of known proteins, multiple homologous sequences were revealed to be present in other GPCRs. Further refinement of the homology with the Psi-BLAST program resulted in the identification of a novel protein domain ~60 residues long found in several dozen recently cloned orphan GPCRs (Fig. 2). The heptahelical cores of the homologous GPCRs were also similar, and all fell into the secretin receptor family (GPCRB family). Thus, this novel extra membrane domain represents the eighth region of significant homology, which is quite unusual for GPCRs. Interestingly, at least seven of these receptors, leukocyte antigen CD97, CIRL-2, CIRL-3, ETL, Flamingo, VLGR-1, IgHepta, and EMR4 are encoded by one gene but consist of two subunits that suggests posttranslational proteolytic processing similar to CIRL (3, 10, 16, 19–22). Thus, it is likely that the cleavage site found in CIRL is present and functionally significant in other homologous GPCRs of the secretin receptor family. We have therefore designated the novel conserved motif that surrounds the CIRL cleavage site as GPS for GPCR proteolytic site (14, 16).

In all homologous GPCRs, the GPS domain is located in the extracellular portion of the receptors immediately adjacent to the first transmembrane segment. The multiple alignment (Fig. 2) identifies four cysteines, including CXC, and two invariable tryptophans, which are perfectly conserved residues of the GPS signature. In other positions there is a strong preference for either small hydrophobic or aromatic residues. Notably, a short stretch of hydrophobic residues is found C-terminal to the cleavage site. The simplest description of GPS consensus would be Cys-Xaa<sub>2</sub>-Trp-Xaa<sub>1–6</sub>-Trp-Xaa<sub>4</sub>-Cys-Xaa<sub>10–22</sub>-Cys-Xaa-Cys.

To test the hypothesis that the GPS domain is of key importance for the proteolytic processing of GPCRs, we generated three mutants of CIRL with single residue substitutions (Fig. 3A). In the first mutant, the residue C-terminal to the cleavage site Thr<sup>R38</sup> was replaced with Pro. In the second, Cys<sup>R34</sup>, which is one of the two neighboring cysteines close to the proteolysis site, was replaced with Trp. In the third, Trp<sup>R15</sup>, quite distant from the cleavage site but highly conserved in the GPS domain, was mutated to Ser.

The mutants were expressed in COS cells and analyzed by Western blotting with anti-p120 and anti-p85 antibodies. The cells transfected with either wild-type or mutated CIRL showed comparable amounts of expressed receptor. However, in contrast with wild-type CIRL, the mutants did not show any evidence of the cleavage (Fig. 3B). To check that the point mutations had not resulted in a major misfolding of the receptor, we also analyzed the expressed proteins for α-latrotoxin binding activity. We had previously shown that CIRL can be functionally expressed in COS cells as a high affinity receptor of α-latrotoxin, and its activity can be verified either by a direct binding assay of the transfected cells with radioactively labeled α-latrotoxin or by immunoprecipitation of the toxin-receptor complexes (7, 14).

The transfected cells were extracted with Triton X-100 and immunoprecipitated with anti-p85 antibody in the presence of 0.2 nM [125I]-α-latrotoxin. All tested cell extracts, except for mock transfected extracts, exhibited significant and similar levels of α-latrotoxin binding activity (Fig. 3C). Western blotting of the immunoprecipitates with anti-p120 antibody showed that only precursors of the mutants were present there without any traces of processed p120 (Fig. 3D). We have shown earlier (14)
that the α-latrotoxin-binding site of CIRL is located in its extracellular domain. Unexpectedly, no significant activity of mutant-transfected cells could be detected when the intact cells were analyzed by the direct α-latrotoxin binding assay (Fig. 3C). Because CIRL mutants interacted with α-latrotoxin quite well in the immunoprecipitation experiment, the simplest explanation of this finding would be that the mutated receptors were not transported to the cell surface. To investigate this possibility further, the transfected cells, either intact or permeabilized, were fixed and stained with the anti-p120 antibody, which reacts with the extracellular region of CIRL and with the anti-p85 antibody (Fig. 3E). A dramatic difference was noted between wild-type and mutant-transfected cell staining. With the mutants, no p120 could be detected in cells unless they were permeabilized. However, the permeabilized cells showed similar expression levels of CIRL as evidenced by p85 staining. Therefore, the mutation of either the cleavage site or distant residues in the GPS domain did not change a known functional property of CIRL but resulted in the complete arrest of its posttranslational proteolysis. Also, the non-cleavable CIRL mutants were not properly expressed at the cell surface.

An explanation of the link between CIRL cell surface expression and its proteolytic processing might be that CIRL is cleaved by a protease that resides extracellularly. In that case, if the mutated receptor could not be transported to the cell surface, it would no longer be accessible to the protease. An alternative possibility is that mutations in the GPS domain may render the receptor insensitive to the protease, and, as a consequence of no cleavage, receptor trafficking would be impaired. To distinguish between these two possibilities and to determine the subcellular localization of the CIRL proteolytic processing, we analyzed in parallel the proteolytic processing and glycosylation of CIRL and its mutants by pulse-chase labeling of exogenously expressed receptor.

CIRL-transfected COS cells were briefly (30 min) incubated with a mixture of [35S]amino acids. After removal of the labeling medium, the cells were incubated with non-labeled medium for 0, 30, 90, or 150 min and lysed with an ice-cold detergent-containing buffer. CIRL was immunoprecipitated from the extracts, digested with PNGase F and endoglycosidase H, and electrophoresed (Fig. 4A). PNGase F removes most N-linked sugar chains, whereas endoglycosidase H removes efficiently the carbohydrates added in the endoplasmic reticulum but does not cleave all complex carbohydrate chains synthesized in the Golgi compartment. The resulting data indicate that the glycosylated CIRL precursor (the 180-kDa band) is cleaved quite early, yielding p120 (the 105-kDa band), which can be deglycosylated by either glycosidase. Starting from the 30 min point, p120 is glycosylated further (the 120-kDa band), and the mature protein becomes partially endoglycosidase H-resistant, indicating that it has been transported to the Golgi apparatus. The mature p120 is also partially resistant to PNGase F, which may be explained by extensive O-linked glycosylation in its serine-, threonine-, and proline-rich (STP) domain. These data suggest that proteolytic processing of CIRL occurs in parallel with early glycosylation in the endoplasmic reticulum and significantly precedes the synthesis of complex endoglycosidase H-resistant sugar chains in the Golgi apparatus.

A similar experiment was performed with the T838P mutant. As anticipated, no proteolytic processing was detected. Otherwise, the pattern of glycosylation of this mutant did not differ significantly from wild-type CIRL. The late addition of carbohydrates was noted (Fig. 4B, marked with an asterisk),
and this product showed partial endoglycosidase H resistance. Thus, the intracellular trafficking of the mutant was essentially similar to the trafficking of proteolyzed wild-type CIRL up to the latest stage when the receptors are transported to the cytoplasmic membrane. However, the intensity of the mutant receptor bands decreased significantly at the later time points as compared with the wild-type CIRL. Apparently, the turnover rate of the mutant receptor is significantly faster than of the wild-type one. This effect may be explained by enhanced degradation of the uncleaved mutant receptors as a result of either their improper fold or trafficking.

DISCUSSION

CIRL (also called latrophilin, lectomedin, and CL) was originally discovered as a target for the presynaptic neurotoxin α-latrotoxin (reviewed in Ref. 23). A study on CIRL overexpression in chromaffin cells suggested that CIRL has a role in the regulation of secretion (24). CIRL has been one of the first examples of receptors that represent natural chimeras of cell adhesion protein and GPCR. Similarly to several other chimeric receptors, CIRL consists of two subunits, one of them with adhesion features and the other resembling a typical GPCR. The two-subunit structure of CIRL results from endogenous proteolytic processing early in the biosynthetic pathway and involves a GPS motif conserved in chimeric adhesion GPCRs. Our results, together with other reports of the chimeric receptors (discussed below), suggest a common mechanism in the proteolytic processing of all GPS-containing adhesion GPCRs.

Our data suggest that the proteolytic processing of CIRL takes place quite early in the biosynthetic pathway, either in the endoplasmic reticulum or in the early compartment of the Golgi apparatus. The same localization of the processing has been reported for CD97 (3) and Ig-Hepta (21), two receptors with homology to CIRL. These data point to a novel mechanism, because most known receptor-processing proteases reside in the late compartment of Golgi.

Several independent experiments indicate that the site of CIRL cleavage lies between residues Leu387 and Thr398 of the precursor. It is located 19 residues from the first transmembrane segment of CIRL in the C-terminal part of the GPS domain that contains a box of four cysteines, two tryptophans, and a stretch of hydrophobic residues. Our data agree well with the recently reported identification of the cleavage sites in Ig-Hepta and EMR4 by N-terminal amino acid sequencing of the fragments C-terminal to the cleavage site (21, 22). The position of the cleavage sites in Ig-Hepta and EMR4 with respect to the GPS motif is similar to CIRL, suggesting the same processing mechanism. The cleavage sites in these proteins are different from the basic recognition sequences typical for known receptor-processing furin-like proteases (25). At this time, the identity of the protease that cleaves in the GPS domain remains unknown.

The GPS domain appears to be conserved in a number of homologous adhesion GPCRs (reviewed in Refs. 1 and 2). Their alignment (Fig. 2) reveals that the residues at the cleavage site are poorly conserved. The residue N-terminal to the cleavage is non-charged and non-aromatic, whereas the C-terminal residue is small and hydrophilic. We therefore explored the possibility that the entire GPS domain may play a role in CIRL proteolytic processing. Indeed, three CIRL mutants with single residue substitutions in the GPS domain appeared to be resistant to the cleavage but still bound α-latrotoxin, indicating their correct expression. In the first mutant, the residue that is C-terminal to the first cleavage site was radically changed to proline. In two other constructs, two conserved residues of the GPS motif, the last cysteine or the second tryptophan, were mutated. All three mutants could not be detected at the cell surface, suggesting that proteolytic processing may regulate their surface expression. The analysis of the trafficking of the T838P mutant by pulse-chase labeling indicated that it can be transported to the Golgi but is prone to a faster degradation that may be functionally linked to its absence from the surface.

The available data on other GPS-containing chimeric receptors support the hypothesis that the GPS motif plays the key role for their intracellular proteolytic processing. Several GPS receptors, including CIRL-2 and CIRL-3 (close homologs of CIRL), CD97, Flamingo, ETL, EMR4, and Ig-Hepta, have been shown to consist of two or more subunits due to proteolytic processing similar to that of CIRL. The orphan receptor EMR1 migrates anomalously on SDS gels, which can also be explained by proteolytic processing (5). Other GPCRs with GPS motif have not been studied sufficiently to exclude the possibility of their cleavage. Very recently, localization of the cleavage sites in Ig-Hepta and EMR4 was reported (21, 22). Their position with respect to the GPS motif is similar to CIRL, suggesting the same processing mechanism. Moreover, CD97 (3) and Ig-Hepta (21) have been shown to cleave in the endoplasmic reticulum in a similar manner to CIRL.

We postulate that the GPS motif defines a novel family of GPCRs, because almost all proteins containing the GPS motif are heptahedral receptors. The only exception is the sea urchin sperm receptor suREJ (26). It has only one transmembrane domain, which is homologous to the first transmembrane segments of the secretin receptor family. This receptor is proteolytically processed, which is likely due to the presence of the GPS motif. It was proposed that the GPS motif is present in two homologs of suREJ, PKDREJ (a putative mammalian ortholog of suREJ), and the polycystic kidney disease protein (PKD1) (27). However, conservation of the GPS motif among homologous GPCRs is much stronger than among suREJ, PKDREJ, and PKD1 (Fig. 2). PKDREJ does not have one of two tryptophans perfectly conserved in GPS GPCRs; its homolog, PKD1, has a sequence resembling GPS but without one of the conserved cysteines. No data are available about their cleavage. Thus, in our opinion, the GPS family should be limited to the homologous adhesion GPCRs. We may speculate that the suREJ gene has resulted from accidental exon shuffling that removed a part of the heptahedral core of a GPS-containing GPCR. Later in evolution, the GPS domain of a non-heptahedral receptor has become rudimentary and deteriorated in PKDREJ and PKD1.

In addition to the presence of GPS motifs, the homologous heptahedral receptors have other common structural features that define them as a family. First, their seven transmembrane segments are homologous and put them in the secretin receptor or GPCRB family. Thus, the members of the GPS subfamily have eight regions of a significant homology that distinguishes them from other heptahedral receptors. Second, these receptors contain large extracellular N-terminal regions that contain variable sets of domains typical for cell adhesion proteins (Fig. 5). CIRL has lectin, olfactomedin-like, and mucin-like STP domains. In other receptors, multiple EGF, IgG, thrombospondin, and cadherin repeats are also found. Thus, the known GPS receptors represent natural chimeras of G protein signaling receptors and cell adhesion molecules.

What might be the functional importance of the proteolytic processing of chimeric receptors? One of the best known examples of GPCR proteolysis is the activation of the thrombin receptors by extracellular cleavage with thrombin. As a result of the cleavage, the receptor internal sequence is unmasked,
working as an endogenous agonist to trigger receptor-mediated G protein signaling (13). The primary proteolysis of CIRL and other GPS receptors does not seem to be a similar case, because it is intracellular and virtually 100% efficient. Therefore, their intracellular cleavage is likely to represent a constitutive event and may be important for the proper trafficking of the GPS receptors.

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