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Association of the subunits of the calcium-independent receptor of α-latrotoxin

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ABSTRACT

CIRL-1 also called latrophilin 1 or CL belongs to the family of adhesion G protein-coupled receptors (GPCRs). As all members of adhesion GPSR family CIRL-1 consists of two heterologous subunits, extracellular hydrophilic p120 and heptahelical membrane protein p85. Both CIRL-1 subunits are encoded by one gene but as a result of intracellular proteolysis of precursor, mature receptor has two-subunit structure. It was also shown that a minor portion of the CIRL-1 receptor complexes dissociates, producing the soluble receptor ectodomain, and this dissociation is due to the second cleavage at the site between the site of primary proteolysis and the first transmembrane domain. Recently model of independent localization p120 and p85 on the cell surface was proposed. In this article we evaluated the amount of p120–p85 complex still presented on the cellular membrane and confirmed that on cell surface major amount of mature CIRL-1 presented as a p120–p85 subunit complex.

1. Introduction

The super-family of G-protein coupled receptors (GPCRs) is one of the largest families of proteins in the human genome. According GRAFS classification systems based on phylogenetic analyses there are five main families named glutamate, rhodopsin, adhesion, frizzled/taste2 and secretin [1]. Adhesion-GPCRs are characterized by existence of a large extracellular N-terminal domain with various cell-adhesion modules (for a review, see [2]). The N-terminal region links to seven-transmembrane-spanning (TM7) intracellular domain via GPSR proteolytic site (GPS)-containing region. This domain was found at the same juxtamembrane position in all homologous adhesion GPSRs with the only exception of GPR123 [3]. GPS is essential in the proteolytic cleavage that occurs immediately after receptor biosynthesis within the endoplasmic reticulum [4,5] or early Golgi [6]. The mature receptor is a noncovalently associated two-subunit complex expressed on the cell surface [7].

It has been recently proposed that two fragments of CIRL p120 and p85 remain membrane bound but dissociate and behave as independent cell surface proteins. It was shown they recycled separately, individually solubilized by perfluorooctanoic acid (PFO) but can re-associate upon the Triton X-100 extraction with further immunoprecipitation [5]. Also recent experiments with several chimeras of CIRL-1 and EMR2 demonstrated that complementary fragments from distinct adhesion GPCRs can cross-interact and produce functionally active cross-complexes [8]. The nature of the p120 independent anchoring in the membrane as well as mechanism of reassociation is still under investigation.

Here we showed that the major amount of mature CIRL-1 presented on cell surface as a p120–p85 subunit complex.

2. Materials and methods

2.1. DNA plasmids and constructions

The plasmid pcDNA HA-CIRL-1 was described earlier [9]. The plasmid pcDNA HA-CIRL-1-EGFP was obtained by in-frame ligation of EGFP-coding sequence (pEGFP-N1, Clontech) into 3′-terminus of HA-CIRL-1 in the plasmid pcDNA HA-CIRL-1. The plasmid pSeqTag 7-6-CIRL-1-EGFP was obtained by in-frame ligation of appropriate p85-EGFP coding sequence from pcDNA HA-CIRL-1-EGFP in the plasmid pSTR7-6 [10]. The construct encoding CIRL mutant with introduced thrombin cleavage site (thr-CIRL) was made by cloning of the PCR product obtained from cDNA CIRL-1 with primers AACTGCTAGATATCCTGGATGCCCAG and AAAAGATCTCTGCGGCCG CAGAACCACGCGGAACCAGTCGGTGAGCCATGAGCACTG into cDNA CIRL at *Eco*RV and *Bgl*II sites. The correct sequence of all constructs obtained by PCR was additionally verified by DNA sequencing.

2.2. Antibodies and immunoblotting

Chicken antibodies specific to p120 and p85 subunits of CIRL-1 were described previously [7,11]. The following antibodies were also used: rabbit anti-HA antibody (Bethyl), chicken anti-GFP antibody (Upstate), mouse anti-Myc antibody (Invitrogen) and goat anti-rabbit, goat anti-mouse and goat anti-chicken HRP-conjugated antibodies (Jackson ImmunoResearch).

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SDS–polyacrylamide gel electrophoresis and Western blotting were performed by standard procedures. Blots were blocked overnight in 5% non-fat milk in 10 mm Tris–HCl, pH 7.8, 150 mm NaCl and 0.1% Tween 20 and then incubated with primary antibodies as indicated in the individual figure legends. After incubation with horseradish peroxidase-conjugated secondary antibodies, immunoreactive bands were visualized by enhanced chemiluminescence.

2.3. Cells and transfection

COS cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2 mM l-glutamine. The cells were transfected with indicated constructs using Unifectin-56 (UnifectGroup).

2.4. PFO treatment

COS cells were transfected with pSTR7-2 [10] or pSTR7-2 T/P [4]. In 3 days the conditioned media were collected, centrifuged at 15,000*g* for 15 min (4 °C) and incubated overnight with WGA-agarose (Sigma). The absorbed on WGA-agarose proteins from cell media were eluted with 20 mM Tris–HCl, pH 7.7, containing 0.5 M *N*-Acetyl-D-glucosamine and 0.5 M NaCl. A 500 mkL aliquots of eluates were diluted with 20 mM Tris–HCl, pH 7.7 to final concentration of 160 mM NaCl and were loaded on Ni–NTA agarose (30 mkL) (Qiagen) in the presence or in the absence of 0.5% PFO (perfluorooctanoic acid, Sigma). The absorbed on Ni–NTA agarose proteins were eluted with 20 mM Tris–HCl, pH 7.7, containing 250 mM imidazole and 150 mM NaCl. The eluates from Ni-agarose were separated by electrophoresis on 10% SDS–polyacrylamide gel followed by Western blotting onto ECL-grade nitrocellulose. The blots were probed with chicken anti-p120 CIRL-1 and mouse anti-Myc antibodies.

2.5. Subunit exchange

For subunit exchange studies COS cells were transfected or cotransfected with pcDNA HA-CIRL-1, pcDNA HA-CIRL-1-EGFP or pSeqTag 7-6-CIRL-1-EGFP. After 30–36 h cells were collected with room temperature PBS, harvested by centrifugation at 600*g* for 1 min and washed three times with PBS the same manner. The cell pellets after last centrifugation were dissolved in 1 mg/ml Sulfo-NHS-LC-Biotin (Pierce)/PBS and incubated at room temperature for 3 h with continuous gentle mixing. The cells were quenched with 100 mM glycine/PBS at room temperature for 5 min, washed with PBS and incubated with anti-HA antibody (1:100) at room temperature for 1 h with continuous gentle mixing. Cells were washed with PBS, harvested by centrifugation at 600 x *g* for 1 min and solubilized with cold lysis buffer (50 mM Tris–HCl, pH 7.3, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF). Cell solubilizates were obtained by centrifugation at 15,000 × *g* for 15 min (4 °C). For protein immunoprecipitation solubilizates were incubated with Protein A agarose (Sigma) and proteins after immunoprecipitation were loaded on an SDS gel, transferred onto nitrocellulose membrane and probed with anti-p85 CIRL-1, anti-GFP antibodies and streptavidin-HRP.

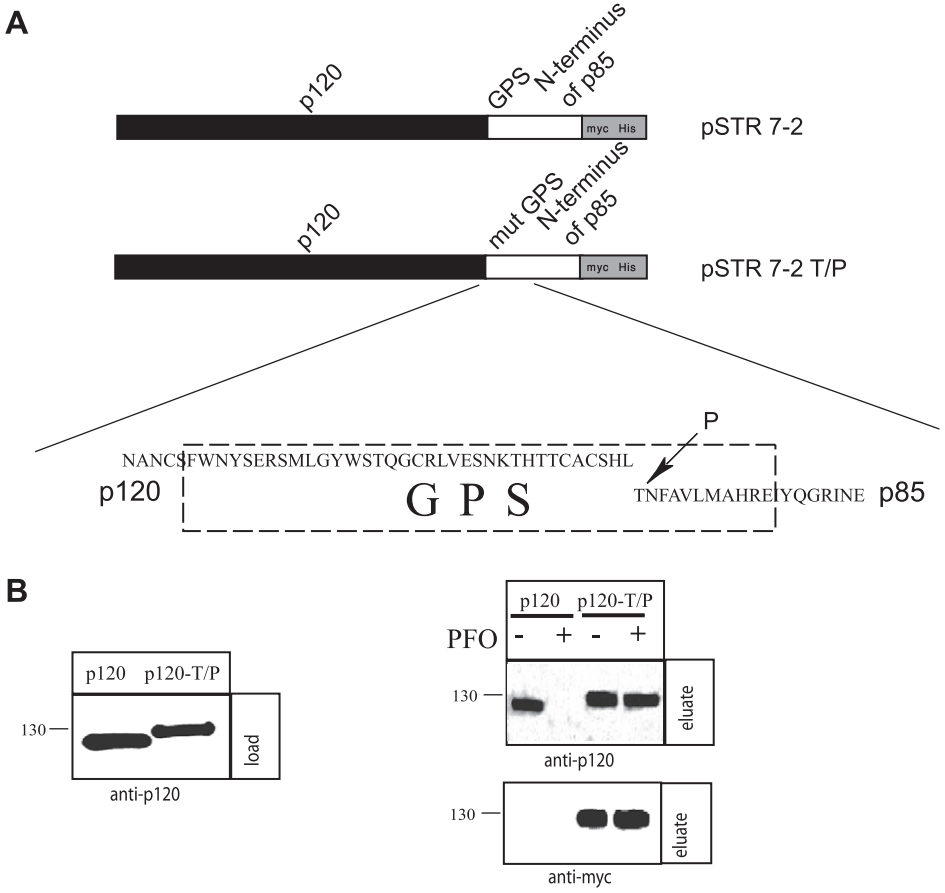

Fig. 1. Complex of p120 and p85 can be extracted from cell media. (A) Schematic description of soluble deletion constructs used herein. (B) COS cells were transfected with the plasmids encoding either soluble wild-type CIRL ectodomain or GPS domain mutant (T_{838}/P). The proteins of conditioned media were concentrated using WGA-agarose followed by precipitated with Ni–NTA agarose in the presence "+" or in the absence "–" of 0.5% PFO. After elution proteins were loaded onto SDS-gel and transferred onto nitrocellulose membrane which was blotted with either anti-p120 or anti-myc antibodies.

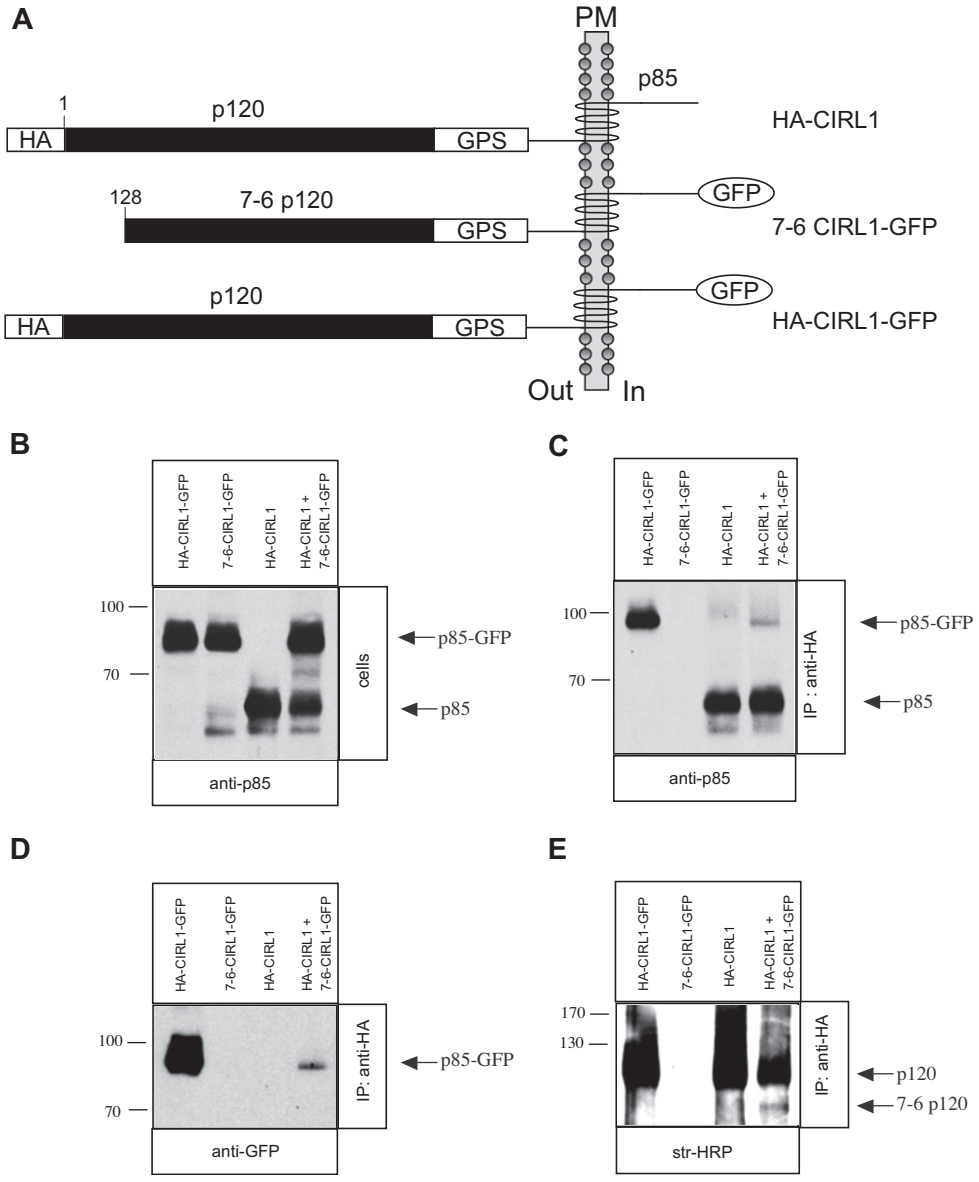

Fig. 2. Cross-immunoprecipitation of co-expressed CIRL-constructs. (A) Schematic description of constructs used in the experiment. (B–E) COS cells were transfected or co-transfected with indicated constructs. After biotinylation cells were incubated with anti-HA antibody followed by cell lysis with Triton X-100-containing buffer. Receptors bound to antibodies were then collected with protein A agarose. The initial extracts (B) and the SDS-eluates after immunoprecipitation (C–E) were analyzed with indicated antibodies shown on the bottom.

2.6. Thrombin treatment

COS cells were transfected with either wild-type CIRL or thr-CIRL mutant, containing thrombin cleavage site in the GPS domain. In 36 h, the transfected cells were washed twice with PBS and further incubated in PBS containing 10 units/ml of thrombin for 40 min at room temperature. The reaction was stopped by the addition of 100 mM phenylmethylsulphonyl fluoride solution in isopropanol to the final concentration of 1 mM. The cell media were collected, centrifuged at 15,000 × *g* for 15 min (4 °C) and incubated overnight with α-latrotoxin-agarose. The cells were lysed in the SDS loading buffer. Proteins absorbed on α-latrotoxin-agarose were eluted with 50 mM Tris–HCl, pH 7.5, containing 1 M KCl. The cell lysates and eluates were separated by electrophoresis on 10% SDS–polyacrylamide gel followed by Western blotting with chicken anti-p120 CIRL-1 antibodies.

3. Results

The hypothesis about independent anchoring of p120 subunit to membrane was initially tested by perfluorooctanoic acid (PFO) solubilization [5]. As at low concentrations (0.1–0.6%) PFO only removed p120 from the cell membrane, leaving all p85 in the cell pellet it was proposed that releasing of p120 was caused by partial solubilization of plasma membrane but not the p120–p85 complex dissociation. To exclude the possibility that PFO could destroy complex between p120 and p85 subunits we used purified CIRL-1 chimeras expressed with two earlier described plasmids pSTR7-2 and pSTR7-2 T/P (see Section 2). pSTR7-2 encodes entire soluble ectodomain of CIRL-1 (p120 and a small extracellular N-terminal fragment of p85 tagged with Myc and 6 × His) and pSTR7-2 T/P contains point mutation (Thr_{838} to Pro) within the GPS cleavage site which inhibits proteolysis of the precursor

(Fig. 1A). After cell transfection proteins from cell media were concentrated on WGA-agarose and after elution were loaded onto Ni–NTA either in the presence or absence of 0.5% PFO (Fig. 1B, at the left). Without PFO p120 of 7-2 CIRL-1 was successfully precipitated by its cognate fragment (Fig. 1B, at the right on top) but PFO presented in buffer prevented precipitation of p120 with p85-fragment by disrupting p120–p85 complex but not affecting protein binding to Ni–NTA (Fig. 1B, p120-T/P).

To clarify the intriguing data about re-association of independent p120 and p85 subunits under detergent solubilization we created several constructs of CIRL-1 with different length of subunits (Fig. 2A). This approach allowed us to discriminate between these subunits on the same blot and to evaluate amount of each form after immunoprecipitation. All fusion proteins were efficiently expressed or co-expressed in COS cells giving the opportunity to evidently distinguish p85 and p85-GFP (Fig. 2B). It should be noted that molecular mass of p85 expressed in eukaryotic cells differs from molecular mass of p85 from rat brains (for instance see in [5]). To precipitate only the surface receptors, intact cells after biotinylation were incubated with anti-HA antibody and then proteins were extracted with Triton X-100.

If p120 was independent membrane-anchored protein and detergent solubilization stimulated re-association of p120 and p85 subunits the "exchange" of complementary fragments would occur as well as p120/p85-GFP and 7-6-p120/p85 chimeras would be generated. Indeed, anti-HA antibody besides p85 also precipitated p85-GFP (Fig. 2C and D) but with efficacy that hardly expected in case of independent subunit behavior. If we take the amount of precipitated p85 as 100%, then only 8% of p85-GFP was co-precipitated. Another explanation for this observation is that oligomerization of HA-CIRL1 and 7-6-CIRL1-GFP in the cell membrane could lead to their co-precipitation (Fig. 2C). In this case 7-6-p120 should also be detected in respective precipitate. Biotinylation performed prior solubilization allowed us to jointly stain precipitated full length and shortened subunits of p120 with streptavidin-HRP (Fig. 2E). Detection of 7-6-p120 precipitated on HA-antibody indicates oligomerization of CIRL-1 receptors.

To directly test if CIRL-1 subunits present on the plasma membrane surface as a complex new construct was designed. We introduced thrombin cleavage site in the N-terminus of p85 C-terminally to the short hydrophobic stretch of the GPS domain, about 14 residues far from the first transmembrane domain (Fig. 3A). The thr-CIRL-1 chimera expressed in COS cells was endogenously cleaved similarly to wild-type CIRL-1, into p120 and p85 subunits (Fig. 3B, top). Then the cells were transiently treated with thrombin and p120 released to the cell medium was collected using α-latrotoxin-agarose. Only p120 from the cells expressing thr-CIRL-1 chimera was observed in the cell medium after thrombin treatment, thus indicating that cleavage on the thrombin site located in p85 subunit caused the release of cognate p120 subunit (Fig. 3B, bottom).

4. Discussion

CIRL-1 is an orphan cell surface receptor expressed in brain. It was originally discovered as a target of potent presynaptic neurotoxin, latrotoxin, and was shown to regulate secretion [12,13]. CIRL was one of the first examples of receptors that represented natural chimeras of cell adhesion protein and GPCR. Similarly to several other adhesion receptors, p120 subunit and TM7 p85 subunit of CIRL-1 are thought to be constitutively non-covalently associated following GPS proteolysis. So CIRL-1 expresses on the cell surface as heterodimer. However, new model of organization and functioning of CIRL-1 was proposed [5]. According to this mechanism the extracellular p120 subunit dissociates from p85, anchors to the cell membrane and functions as an independent protein, and reassociation of the subunits occurs under solubilization or latrotoxin binding to p120. This model was based mostly on observation of cross-precipitation of chimeras of CIRL-1 with different tags on p120 or p85 subunits [5]. Also experiments with chimeras of CIRL-1 and EMR2 demonstrated that complementary fragments from distinct adhesion GPCRs can cross-interact and produce functionally active cross-talking complexes [8]. All these observations can be interpreted by oligomerization of the receptors. Our recent findings indicate that as a result of second proteolysis p120 dissociates from the cell membrane [14]. Hence p85 subunit can be presented on cell membrane without p120 and oligomerize with full length CIRL receptors. This model of oligomerization of different chimeras of CIRL-1 can explain previous results of cross-precipitation and cross-talking of receptors.

As it is fundamental issue for understanding the structure and function of adhesion GPSRs, the series of clarification experiments were performed. At first we tested if complex of mature subunits p120–p85 was still present under native conditions. Using soluble

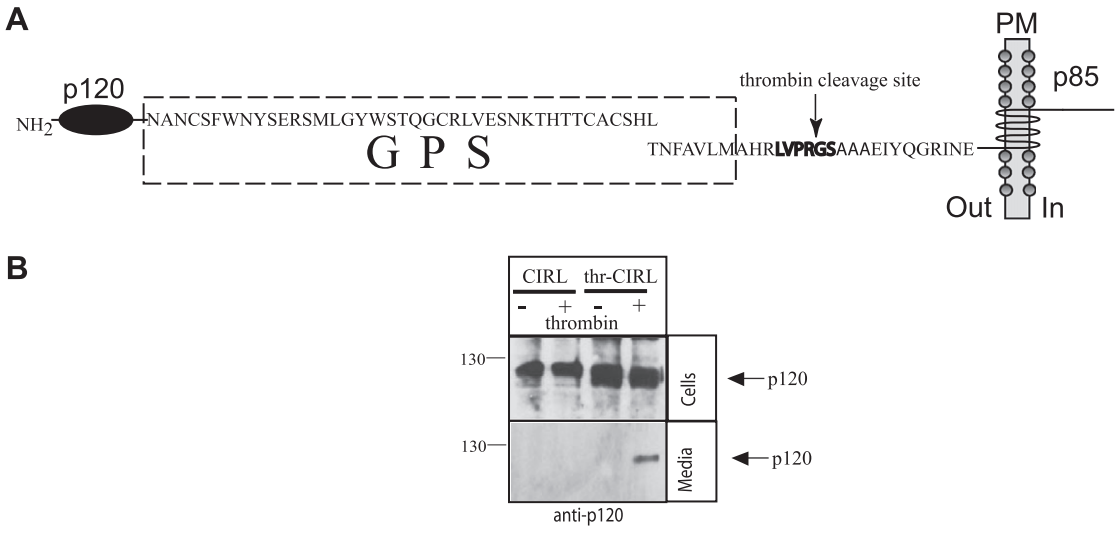

Fig. 3. Thrombin cleavage within p85 results in p120 release. (A) Schematic description of constructs used herein. (B) COS cells after transfection were incubated with thrombin and proteins of cell media were concentrated with α-latrotoxin-agarose. Eluates and total cell proteins were transferred onto nitrocellulose membrane which was blotted with indicated antibodies.

form of CIRL-1, 7-2 CIRL-1 (Fig. 1A), with 7TM-less N-terminal fragment of p85, we were able to detect some p120 subunit in complex with p85 fragment in cell media (Fig. 1B). Our findings also indicate that perfluorooctanoic acid (PFO) destroys this complex.

It was then estimated the amount of p120 and p85 that was still present on cell surface as a heterodimer. Constructs of CIRL-1 with different molecular weight of subunits (Fig. 2A) were made to discriminate between these subunits on the same blot and to evaluate amount of each form after immunoprecipitation following co-transfection. The design of experiment was also allowed us to precipitate only cell surface receptors. One of the expected outcomes of this experiment is that in case of truly independent subunit behavior we would detect equiprobable subunit exchange after detergent solubilization followed by immunoprecipitation. Only minor "subunit exchange" was observed and this phenomenon was obtained mainly due to receptor oligomerization (Fig. 2).

These observations raise the question if this complex presents *in vivo*, because all previous results were obtained using transfected cells. It should be noted, that after second cleavage p120 [14] is still associated with small N-terminal fragment of p85 and this peptide was found in rat brain extracts as well as in the conditioned media of CIRL-1-transfected cells. These data point at complex of p120 with p85 *in vivo*. We also demonstrated that some amount of CIRL-1 was still presented on the cell membrane as a p120–p85 complex since cleavage of the thrombin site, located in the extracellular part of p85 subunit, resulted in release of p120 subunit (Fig. 3).

Thus, the data strongly indicate that major amount of mature CIRL-1 present on cell surface as a p120–p85 subunit complex and p120 dissociation can be result of secondary cleavage between G852 and R853 located in extracellular part of p85 [14].

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