Interaction of the α1B-Adrenergic Receptor with gC1q-R, a Multifunctional Protein*

(Received for publication, December 30, 1998, and in revised form, April 7, 1999)

Zhaojun Xu‡, Akira Hirasawa, Hitomi Shinoura, and Gozoh Tsujimoto§
From the Department of Molecular, Cell Pharmacology, National Children's Medical Research Center, Taishido 3-35-31, Setagaya-ku, Tokyo, 154-8009 Japan

gC1q-R, a multifunctional protein, was found to bind with the carboxyl-terminal cytoplasmic domain of the α1B-adrenergic receptor (173 amino acids, amino acids 344–516) in a yeast two-hybrid screen of a cDNA library prepared from the rat liver. In a series of studies with deletion mutants in this region, the ten arginine-rich amino acids (amino acids 369–378) were identified as the site of interaction. The interaction was confirmed by specific co-immunoprecipitation of gC1q-R with full-length α1B-adrenergic receptors expressed on transfected COS-7 cells, as well as by fluorescence confocal laser scanning microscopy, which showed co-localization of these proteins in intact cells. Interestingly, the α1B-adrenergic receptors were exclusively localized to the region of the plasma membrane in COS-7 cells that expressed the α1B-adrenergic receptor alone, whereas gC1q-R was localized in the cytoplasm in COS-7 cells that expressed gC1q-R alone; however, in cells that co-expressed α1B-adrenergic receptors and gC1q-R, most of the α1B-adrenergic receptors were co-localized with gC1q-R in the intracellular region, and a remarkable down-regulation of receptor expression was observed. These observations suggest a new role for the previously identified complement regulatory molecule, gC1q-R, in regulating the cellular localization and expression of the α1B-adrenergic receptors.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Cloning—All components were purchased from CLONTECH, and all assays were carried out as suggested by the manufacturer. The Matchmaker™ two-hybrid system was used to screen a rat liver cDNA library (complexity ~3 × 10^6 total recombinants) constructed in pGAD10 (CLONTECH) with the carboxyl-terminal cytoplasmic domain of the hamster α1B-adrenergic receptor. Bait plasmids were constructed in pGBT9 (CLONTECH) using the carboxyl-terminal cytoplasmic domain of the hamster α1B-adrenergic receptor.

We conducted a search for novel proteins that interact with the α1B-adrenergic receptor, specifically focusing on the carboxyl-terminal cytoplasmic domain, because mutations within this domain have pleiotropic effects on receptor physiology (11–14). Using interaction cloning and biochemical techniques, we demonstrate that gC1q-R interacts with α1B-adrenergic receptors in vitro and in vivo through the specific site and that in cells that co-express α1B-adrenergic receptors and gC1q-R, the subcellular localization of α1B-adrenergic receptors is markedly altered and its expression is down-regulated. These results suggest that gC1q-R plays a role in the regulation of the subcellular localization as well as the function of α1B-adrenergic receptors.
and enzyme-linked chemiluminescence (ECL, Amersham Pharmacia Biotech).

**Confocal Laser Scanning Microscope Analysis**—Carboxyl-terminal green fluorescent protein (GFP)-tagged gC1qR71/GFP was constructed and subcloned into the pEGFP-N3 vector (CLONTECH). Either a carboxyl-terminal FLAG-tagged full-length α1B-adrenergic receptor (α1B-AR), mature form (gC1qR71) (numbers denote amino acid residues in the gC1q-R sequence), B, or the DNA that encodes the α1B-adrenergic receptor carboxyl-terminal tail was inserted into the yeast expression plasmid pGBT9. Each gC1qR fragment indicated in A was inserted into the yeast expression plasmid pGAD424 and co-expressed with the α1H-adrenergic receptor carboxyl-terminal tail in the SFY526 strain. After incubation, the level of β-galactosidase activity was estimated by the liquid culture method with o-nitrophenyl β-D-galactopyranoside as the substrate. Each value represents the mean ± S.D. of three independent experiments. AR, adrenergic receptor.

**RESULTS AND DISCUSSION**

The yeast two-hybrid system (17, 18) was used to identify candidate cellular proteins that interact with the carboxyl-terminal cytoplasmic tail of the hamster α1B-adrenergic receptor. The screening of approximately 2 × 10^7 transformants resulted in the isolation of eleven independent clones that interacted specifically with the carboxyl-terminal cytoplasmic tail of the α1B-adrenergic receptor. Sequence analysis revealed that all eleven clones encoded the same polypeptide, gC1qR (EBI data base AJ001102) (19, 20); two clones encoded the full-length gC1q-R, whereas the remaining nine clones encoded gC1q-R from the 26th residue to beyond the stop codon.

**Flow Cytometry Analysis**—To construct the carboxyl-terminal FLAG/GFP-tagged α1B-adrenergic receptor, the stop codon of the sequence that encodes the α1B-adrenergic receptor was altered to a KpnI site by site-directed mutagenesis PCR, and a FLAG/GFP fragment was cloned at the carboxyl-terminal KpnI site of the α1B-adrenergic receptor. This was subsequently subcloned into the mammalian expression vector pME18s. To construct β-galactosidase/pME18s, the full-length fragment of β-galactosidase from the plasmid pCMV-SPORT β-gal (Life Technologies, Inc.) was inserted into the vector pME18s. α1B-adrenergic receptor-FLAG/GFP and either the gC1qR71 or β-galactosidase expression construct were co-transfected into COS-7 cells by the electroporation method using Cell-Porator (Life Technologies, Inc.) according to the manufacturer's instructions, and the cells were assayed. Forty-eight hours after transfection, the cells were analyzed with FACScan flow cytometry for excitation of GFP (Becton Dickinson, Mountain View, CA) as described previously (16). Routinely, data from fluorescence of 10^4 cells were subjected to two-dimensional dot-plot analysis with FL-1 and FL-3 (Fig. 5A); GFP-positive cells were further gated, and the average value of FL-1 fluorescence intensities of these cells was calculated by the Cell Quest software (Becton Dickinson, Mountain View, CA).

**Fig. 1.** Interaction of the α1H-adrenergic receptor with gC1qR in the yeast two-hybrid system. A, constructs of gC1q-R: the full-length form (gC1qR), prepro form (gC1qR1), and mature form (gC1qR71) (numbers denote amino acid residues in the gC1q-R sequence); B, the DNA that encodes the α1B-adrenergic receptor carboxyl-terminal tail was inserted into the yeast expression plasmid pGBT9. Each gC1qR fragment indicated in A was inserted into the yeast expression plasmid pGAD424 and co-expressed with the α1H-adrenergic receptor carboxyl-terminal tail in the SFY526 strain. After incubation, the level of β-galactosidase activity was estimated by the liquid culture method with o-nitrophenyl β-D-galactopyranoside as the substrate. Each value represents the mean ± S.D. of three independent experiments. AR, adrenergic receptor.

**Fig. 2.** Interaction of the α1H-adrenergic receptor with gC1qR. COS-7 cells were transfected with the expression vector gC1qR (lane 1) or gC1qR71 (lanes 3–6) with (lanes 1–4) or without α1H-adrenergic receptor (lanes 5 and 6). Forty-eight hours after transfection, the cells were harvested and lysed. Extracts were immunoprecipitated with (lanes 1, 3, and 5) or without (lanes 2, 4, and 6) anti-α1H-adrenergic receptor polyclonal antibody at 4 °C overnight. The samples were then subjected to SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and probed with anti-HA monoclonal antibody. The position of the molecular mass standard in kDa is shown on the left. AR, adrenergic receptor.
gC1q-R open reading frame encodes a prepro-protein of 279 amino acid residues (Fig. 1A). The mature protein is preceded by a 13-residue-long leader peptide, which probably contains the signal peptide. The precise function of the 57 residues immediately preceding the mature protein has not been determined, but is predicted to play a role in cellular translocation. The mature protein is presumed to be generated by site-specific cleavage and removal during post-translational processing. We, therefore, constructed the prepro form of gC1q-R (gC1qR), the amino terminus fragment cleaved from prepro gC1q-R (gC1qR1), and the mature form of gC1q-R (gC1qR71) by PCR and compared the binding of each construct with the carboxyl-terminal cytoplasmic tail of the $\alpha_{1B}$-adrenergic receptor in the yeast two-hybrid assay. As shown in Fig. 1B, both the prepro form and mature form of gC1q-R, but not the amino-terminal fragment, were found to interact with the $\alpha_{1B}$-adrenergic receptor; however, the prepro form of gC1q-R (gC1qR) interacted with the $\alpha_{1B}$-adrenergic receptor to a much lesser degree than the mature form of gC1q-R (gC1qR71).

We next examined the interaction between the $\alpha_{1B}$-adrenergic receptor and gC1q-R in vivo. Both gC1qR and gC1qR71 were epitope-tagged with HA at the carboxyl terminus (gC1qR/HA and gC1qR71/HA), and the $\alpha_{1B}$-adrenergic receptor was transiently co-expressed with either gC1qR/HA or gC1qR71/HA in COS-7 cells. The cell lysate was then immunoprecipitated with the anti-$\alpha_{1B}$-adrenergic receptor polyclonal antibody and subjected to Western blot analysis with anti-HA antibody (Fig. 2). Anti-$\alpha_{1B}$-adrenergic receptor antibody coprecipitated gC1qR and gC1qR71 only in the cells that co-expressed the $\alpha_{1B}$-adrenergic receptor, demonstrating in vivo that the $\alpha_{1B}$-adrenergic receptor interacts with gC1q-R (Fig. 2). Furthermore, in the cells transfected with the full-length gC1q-R cDNA and in the cells transfected with gC1qR71, a 32-kDa protein was detected, indicating that the HA-tagged
prepro-gC1q-R was efficiently processed to the mature form in the COS-7 cells (Fig. 2). Taken together with the results from the yeast two-hybrid assay (Fig. 1) and immunoprecipitation analysis (Fig. 2), the mature form of gC1q-R (gC1qR71) rather than prepro-gC1q-R (gC1qR) is considered to bind more efficiently with the \( \alpha_{1B} \)-adrenergic receptor. Therefore, in the following experiments, we examined the interaction of gC1qR71 with the \( \alpha_{1B} \)-adrenergic receptor.

Furthermore, to search the site of interaction within the carboxyl-terminal cytoplasmic tail of the \( \alpha_{1B} \)-adrenergic receptor (\( \alpha_{1B}-AR \)), which interacts with gC1qR71, we constructed a series of deletion mutants of the carboxyl-terminal cytoplasmic tail of the \( \alpha_{1B} \)-adrenergic receptor. As shown in Fig. 3A, the carboxyl-terminal cytoplasmic tail of the hamster \( \alpha_{1B}-AR \) contains the NPXY motif at amino acids 344–348, which is highly conserved among G protein-coupled receptors, the putative acidic/dihydrophobic sequence motif (amino acids 349–364), which was recently shown to mediate cell surface delivery of a vasopressin receptor (21), and the arginine-rich region (amino acids 371–378). Thus, we constructed thirteen truncated forms of the \( \alpha_{1B} \)-adrenergic receptor carboxyl-terminal tail as shown in Fig. 3A and compared the binding of each construct with gC1qR71 in the yeast two-hybrid assay. As shown in Fig. 3B, gC1qR71 could bind with the \( \alpha_{1B} \)-adrenergic receptor carboxyl-terminal tail that contains the arginine-rich region.

Next, we examined the subcellular localization of the \( \alpha_{1B} \)-adrenergic receptor (FLAG-tagged \( \alpha_{1B} \)-adrenergic receptor) using a fluorescent antibody, as well as the endogenous fluorescence (gC1qR71/GFP) by fluorescent confocal microscopy. As seen in Fig. 4 (A, C, E, H, and K) immunocytochemical analysis of cells transiently transfected with only FLAG-tagged \( \alpha_{1B} \)-adrenergic receptor showed that the fluorescence distribution was typical of that of a plasma membrane-labeling pattern.
The yeast two-hybrid assays that gC1qR71 could decrease the fluorescent signal of FLAG-tagged a1B-adrenergic receptor. The mean value of fluorescence intensity in each experimental condition. The values represent the mean ± S.D. of at least three independent experiments. AR, adrenergic receptor.

| Table I Effect of gC1q-R on a1B-adrenergic receptor binding |  |
| --- | --- | |  |
| COS-7 cells were transfected with a1B-adrenergic receptor alone or co-transfected with a1B-adrenergic receptor and either gC1qR71 or β-galactosidase. Forty-eight hours after transfection, cells were harvested and analyzed by a fluorescence-activated cell sorter flow cytometer. A, results are shown as a two-dimensional dot plot of GFP fluorescence. GFP+, cells expressing GFP-fused a1B-adrenergic receptor; GFP−, cells that do not express GFP-fused a1B-adrenergic receptor. The x axis represents the relative fluorescence of GFP. B, the mean value of fluorescence intensity in each experimental condition. The values represent the mean ± S.D. of at least three independent experiments. AR, adrenergic receptor. |  |

| B (pmol/mg protein) | a1B-AR | a1B-AR/β-galactosidase | a1B-AR/gC1qR71 |
| --- | --- | --- | --- |
| α1B-AR | 5.3 ± 1.2 | 4.3 ± 1.5 | 0.7 ± 2.6 |

Using the carboxyl-terminal FLAG/GFP-tagged a1B-adrenergic receptor (α1B-adrenergic receptor/GFP), we further examined the effect that gC1qR71 has on the subcellular localization of the α1B-adrenergic receptor upon co-transfection. GFP fusion in this manner does not perturb normal ligand binding nor the subcellular localization of α1B-adrenergic receptor (22). Flow cytometry analysis of GFP fluorescence enables us to detect the α1B-adrenergic receptors. Cells transfected with the α1B-adrenergic receptor/GFP and gC1qR71/HA were examined by flow cytometry. In cells transfected with the α1B-adrenergic receptor/GFP alone and in cells co-transfected with the α1B-adrenergic receptor/GFP and gC1qR71/HA, approximately 10–20% of the COS-7 cells were positively detected as having GFP-associated fluorescence, indicating successful transfection with α1B-adrenergic receptor/GFP (Fig. 5A). However, as shown in Fig. 5A, the GFP fluorescence of cells, which co-expressed the α1B-adrenergic receptor/GFP and gC1qR71, was significantly lower than the GFP fluorescence of cells, which expressed only α1B-adrenergic receptor/GFP. The mean value of the fluorescence intensity of GFP in cells co-expressing α1B-adrenergic receptor/GFP and gC1qR71 was 87% lower than that in the cells expressing only the α1B-adrenergic receptor-GFP (Fig. 5B). Additionally, the mean value of the fluorescence intensity of α1B-adrenergic receptor-GFP in cells that co-expressed β-galactosidase as a negative control did not differ significantly from that of cells expressing α1B-adrenergic receptor/GFP alone (Fig. 5B). To further assess the effect that gC1qR71 has on the level of expression of the α1B-adrenergic receptor upon co-transfection, a radioligand binding assay was performed on COS-7 cells transiently expressing the α1B-adrenergic receptor with or without gC1qR71. Membrane preparations of these cells were used to determine the saturation binding isotherms for 125I-labeled HEAT (Table I). Although the Kd value was not significantly altered by co-expression of α1B-adrenergic receptor with gC1qR71, the Bmax value of the α1B-adrenergic receptor dramatically decreased when co-transfected with gC1qR71 (Table I).
I). These results are in agreement with the results from the flow cytometry analysis in that the level of expression of α_{1B} adrenergic receptors on the cell surface is lower in the cells co-expressing gC1qR71. Co-expression of β-galactosidase did not significantly affect the 125I-labeled HEAT binding site (Table I).

In this study, we identified a novel cellular protein that interacts with the α_{1B}-adrenergic receptor, gC1q-R. Expression studies indicated that gC1q-R regulates the expression level and cellular localization of the α_{1B}-adrenergic receptor through its carboxyl terminus. gC1q-R was previously identified as a protein that binds to the globular heads of C1q. Recent accumulating evidence suggests that gC1q-R is a multiligand-binding, multifunctional protein with affinity for diverse ligands including thrombin, vitronectin, and high molecular weight kinogen (23, 24). Moreover, the gC1q-R molecule was found to be identical with the splicing factor SF-2 and with a protein that interacts with the human immunodeficiency virus, type I Tat transactivator designated the Tat-associated protein or TatIP (25, 26); however, the biological function of gC1q-R has not been clearly defined. Our present results suggest a new role for the previously identified complement regulatory molecule, gC1q-R, in the regulation of the cellular localization and expression of the α_{1B}-adrenergic receptor.

The carboxyl-terminal cytoplasmic region of the adrenergic receptor has a pleiotropic function, because mutations within this region affect receptor physiology (11–14) and because several domains within this region have been shown to interact with several classes of cytoplasmic proteins. One domain that is conserved in both α_{2} and β_{2}-adrenergic receptors, the carboxyl-terminal DFRXXFXXXX motif, interacts with the α-subunit of the eukaryotic initiation factor 2B (9). This protein interaction enhances the β_{2}-adrenergic receptor-mediated activation of adenyl cyclase (9). The glutamate/dileucine sequence motif conserved in many G protein-coupled receptors is involved in the cell surface transport of receptors (21). Also, the carboxyl terminus of the β_{2}-adrenergic receptor interacts with the Na+/K+ exchange regulatory factor family of PDZ proteins (10). Our study demonstrated that gC1q-R binds with the carboxyl tail of the α_{1B}-adrenergic receptor at the arginine-rich region, which differs from the known domains described above, and that gC1q-R regulates the cellular localization and expression of the α_{1B}-adrenergic receptor through their interaction. Further studies to clarify the functional significance of this protein interaction in the regulation of α_{1B}-adrenergic receptor signaling would be of value.

Acknowledgments—We thank Dr. Susanna Cotececia (Institut de Pharmacologie et Toxicologie, Lausanne, Switzerland) for the generous gift of the cDNA-encoding hamster α_{1B}-AR.