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Receptor Isoform-Specific Interaction of Prostaglandin EP3 Receptor with Muskelin

Hiroshi Hasegawa, Hironori Katoh, Hirotada Fujita, Kazutoshi Mori, and Manabu Negishi[1]
Laboratory of Molecular Neurobiology, Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

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By using the yeast two-hybrid system, muskelin was found to bind with the carboxy-terminal tail of the prostaglandin EP3 receptor α isoform but not with either the β or γ isoform. A direct interaction between the carboxy-terminal tail of the α isoform and muskelin was confirmed *in vitro* using recombinant fusion proteins. Analysis by confocal microscopy indicated that the isoform and muskelin were distributed at the plasma membrane in transfected cells. When the isoform was stimulated by agonist, the receptor was internalized in the cells expressing the receptor alone, but this internalization was partially inhibited by the cotransfection with muskelin. Furthermore, muskelin enhanced the Gi activity of the isoform. Thus, muskelin appears to be an isoform-specific anchoring protein for the EP3 receptor. © 2000 Academic Press

Key Words: **prostaglandin; EP3; G-protein-coupled receptor; muskelin; carboxy-terminal; two-hybrid system; internalization.**

Prostaglandin (PG) E_2 exhibits a broad range of biological actions in diverse tissues through its binding to specific receptors on plasma membrane (1). PGE receptors are pharmacologically subdivided into four subtypes, EP1, EP2, EP3, and EP4, on the basis of their responses to various agonists and antagonists (2, 3). Among the four subtypes, the EP3 subtype has been most well characterized and involved in diverse PGE_2 actions (3). We have cloned the EP3 receptor and demonstrated that this receptor is a G protein-coupled receptor that is coupled to both Gi and G13 linking to inhibition of adenylate cyclase and activation of a small GTPase Rho, respectively (4–6). Furthermore, we have identified the three isoforms of EP3 receptor with different carboxy (C)-terminal tail, EP3α, EP3β, and EP3γ, which are produced through alternative splicing (7, 8). Our previous studies revealed that these isoforms differed in agonist-dependent desensitization (9) and in agonist-independent constitutive Gi activity (10, 11). Furthermore, we recently found that three isoforms showed differential membrane targeting dependent on their C-terminal tail structures (12). However, for the EP3 isoforms it remains unclear if specific proteins exist that are responsible for anchoring or subcellular targeting of the isoforms. We have addressed this issue by searching for proteins that interact with the intracellular C-terminal tails of the isoforms, and we here demonstrate that the C-terminal tail of the α isoform interacts specifically with muskelin.

MATERIALS AND METHODS

Yeast two-hybrid screen. cDNA fragments encoding the C-terminal tails of rat EP3α (amino acid residues 336–365), EP3β (amino acid residues 336–361), EP3γ (amino acid residues 336–364), and rat EP1 (amino acid residues 358–405) were cloned into the yeast bait vector pAS2-1 (CLONTECH). After transformation with the vector of the C-terminal tail of EP3α into the yeast reporter strain Y190, a rat brain cDNA library in the prey vector pACT2 (CLONTECH) was screened for interacting proteins according to the manufacturer's instructions. Out of a total of 3×10^6 transformants, one positive clone was obtained.

For construction of myc-tagged muskelins, cDNA of full-length, N-terminal-half (amino acid residues 1–280) or C-terminal-half (amino acid residues 281–735) muskelin was cloned in frame into a pcDNA3 containing myc-epitope tag sequence at the 5′-end.

In vitro binding assay. The cDNA fragments encoding the C-terminal tails of EP3α and EP3γ were subcloned into pGEX-4T-2 (Amersham Pharmacia Biotech), and the plasmids were transformed into *E. coli* strain BL21, allowing expression of the C-terminal tails as a glutathione S-transferase (GST) fusion protein. Fusion proteins were purified using glutathione-Sepharose beads. For *in vitro* binding assay, GST or GST-C-terminal tail proteins were immobilized on glutathione-Sepharose beads for 10 min at 4°C, and they were incubated for 2 h at 4°C with lysates prepared from human embryonic kidney (HEK) 293 cells transiently transfected with myc-muskelin. Proteins bound to the resin were separated by 7.5% SDS–

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Abbreviations used: PG, prostaglandin; C, carboxy; GST, glutathione S-transferase; HEK, human embryonic kidney; PBS, phosphate-buffered saline; TSP-1, thrombospondin-1; IAP, integrin-associated protein.

[1] To whom correspondence should be addressed. Fax: +81-75-753-7688. E-mail: mnegishi@pharm.kyoto-u.ac.jp.

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A

Gal4-BD hybrid	Gal4-AD hybrid	β-Gal assay
None	Muskelin	−
EP3α-C-tail	Muskelin	+++
EP3β-C-tail	Muskelin	−
EP3γ-C-tail	Muskelin	−
EP1-C-tail	Muskelin	−

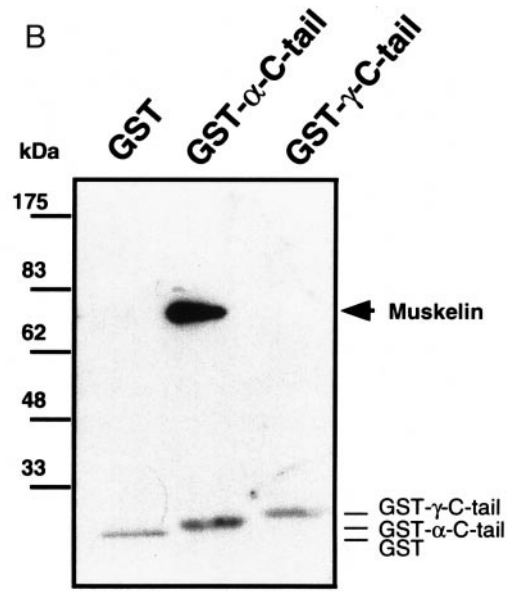

FIG. 1. Interaction of muskelin with the C-terminal tail of EP3α isoform. (A) Yeast two-hybrid system. Yeast reporter strain Y190 expressing the indicated C-terminal tail-fused GAL4-BD was transfected with GAL4-AD-muskelin-hybrid vector, and the relative strength of the interaction was determined by visual inspection of β-galactosidase filter lift assay. +++, very strong; −, negative. (B) *In vitro* binding assay. Lysates of the cells transfected with myc-muskelin were incubated with each GST-fusion protein immobilized on beads. Bound myc-muskelin was visualized with immunoblotting using 9E10 as described under Materials and Methods. The arrow indicates muskelin band.

polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Millipore). Muskelin was detected with an anti-myc antibody (9E10) using an enhanced chemiluminescence ECL Western-blotting system (Amersham Pharmacia Biotech).

Immunofluorescence. HEK 293 cells (1.5×10^5 cells/well) were cultured on poly-D-lysine (Sigma)-coated glass coverslips (circular, 18 mm) in 12-well plates for 1 day. Then cells were transfected with plasmids using LipofectAMINE 2000 (Life Technologies Inc.) according to the manufacturer's instructions and cultured for 2 days. For immunofluorescence, all steps were carried out at room temperature, and cells were rinsed with phosphate-buffered saline (PBS) between each step. After cells had been fixed with 4% paraformaldehyde/PBS for 15 min, they were permeabilized in 0.2% Triton X-100/PBS for 10 min, and incubated with 10% fetal bovine serum in PBS for 30 min. For detection of myc-tagged muskelin and EP3α isoform, cells were incubated with an anti-myc monoclonal antibody 9E10 and a rabbit polyclonal anti-EP3 receptor antibody (13) in PBS for 1 h, followed by the incubation with a rhodamine-conjugated goat anti mouse IgG (Chemicon International Inc.) and a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Chemicon International Inc.). Cells on coverslips were mounted in 90% glycerol containing 0.1% *p*-phenylenediamine dihydrochloride in PBS, and photographed under a laser scanning confocal microscope (Bio-Rad, MRC-1024).

Gi activity and PGE_2 binding assay. For Gi activity of EP3α isoform, HEK 293 cells (1×10^5 cells/well) were transfected with Gs-coupled EP2 and Gi-coupled EP3α, and cultured for 1 day in 24-well plates. After 10 min of incubation at 37°C with 1 μM butaprost (EP2 agonist) and 0.1 μM M&B28767 (EP3 agonist), the cAMP contents of the cells were measured by radioimmunoassay with an Amersham cAMP assay system (Amersham Pharmacia Biotech), as described previously (14). The values are expressed as a percentage of control obtained with the cells stimulated with 1 μM butaprost alone.

For determination of the internalization level of EP3α isoform, cells were transfected with EP3α, and cultured for 1 day in 150-mm dishes. After collected cells (1×10^6 cells/assay) had been incubated for 30 min at 37°C with 20 nM [^{3}H]PGE_2 (200 Ci/mmol; NEN Life Science Products, Inc.), they were then washed and incubated for 10 min at 37°C with or without 20 mM sodium acetate (pH 3.5) and 150 mM NaCl to remove [^{3}H]PGE_2 bound to the receptor remained on the plasma membrane. The specific [^{3}H]PGE_2 binding to the receptor was determined as described previously (9). The values of [^{3}H]PGE_2 bound to the internalized receptor were obtained from the cells treated with sodium acetate and expressed as a percentage of the values of the untreated cells exhibiting the total numbers of the receptors internalized and remained on the plasma membrane.

RESULTS

The C-terminal tail of EP3α isoform was used as bait in a yeast two-hybrid screen of a rat brain cDNA library. In this screen one positive clone was obtained, which was identified as rat muskelin (AB046442), which is 99% identical to mouse muskelin (15) at amino acid level. The specificity of the interaction between the C-terminal tail of EP3α and muskelin was analyzed in the yeast two-hybrid system. Muskelin specifically interacted with the C-terminal tail of EP3α but not with those of other EP3 isoforms or EP1 (Fig. 1A). In order to confirm the interaction observed in the yeast system in terms of true protein–protein interactions, we performed a pull down assay with GST-fusion proteins of the C-terminal tails. Muskelin was recovered with GST-C-terminal tail of EP3α but not with GST-C-terminal tail of EP3γ or GST alone (Fig. 1B), supporting that muskelin specifically interacts with the C-terminal tail of EP3α isoform. We could not obtain GST-C-terminal tail of EP3β due to its incorpo-

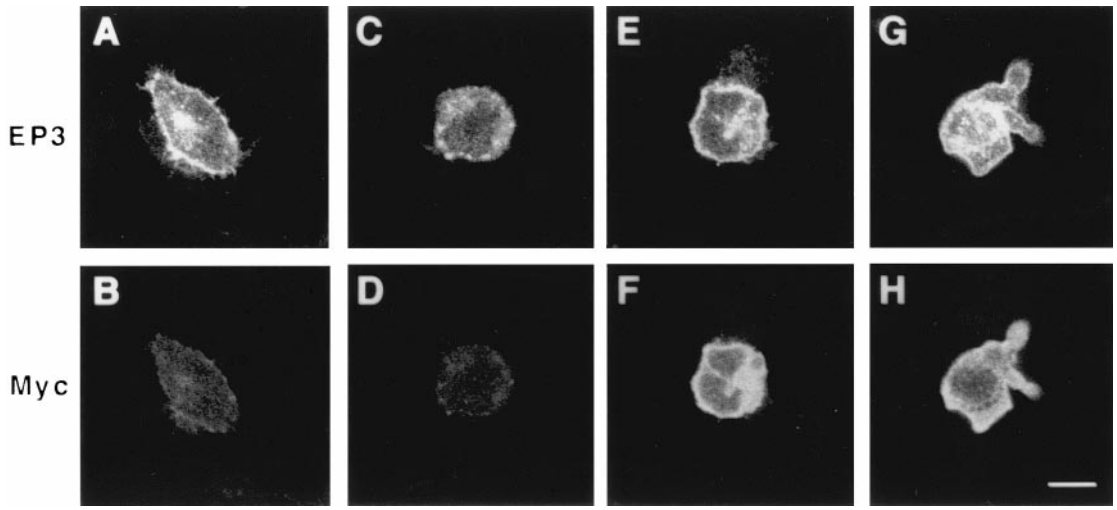

FIG. 2. Effect of muskelin on the internalization of EP3α isoform. After cells had been transfected with EP3α isoform (A–D) or together with myc-muskelin (E–H), they were treated for 30 min with (C, D, G, H) or without (A, B, E, F) 1 μM sulprostone, an EP3 agonist. They were fixed and double-stained with anti-EP3 antibody (A, C, E, G) and 9E10 (B, D, F, H). Bar, 20 μm.

ration into inclusion body of *E. coli*. The binding of muskelin to full-length of EP3α isoform *in vivo* was tried to be analyzed by coimmunoprecipitation from transfected HEK 293 cells. However, we failed to immunoprecipitate the muskelin-receptor complex probably due to disruption of the interaction by detergent (data not shown).

The subcellular localization of myc-tagged muskelin and EP3α isoform was examined in transfected HEK 293 cells by fluorescence microscopy. EP3α isoform was localized at the cell surface, and the isoform was internalized into the intracellular compartment when the cells were stimulated with an EP3 agonist, M&B28767 (Figs. 2A–D). On the other hand, muskelin was visualized in a diffuse cytoplasmic distribution with a slightly intensive staining at the cell periphery. When EP3α isoform was cotransfected with muskelin, the agonist-induced internalization of the isoform was partially suppressed by muskelin (Figs. 2E–H). To assess more directly the internalization of the isoform, we traced the [^{3}H]PGE_2-bound isoform internalized within the cells. Muskelin cotransfection markedly reduced the level of [^{3}H]PGE_2-bound isoform within the cells, indicating that muskelin inhibits the internalization of the isoform (Table 1). Muskelin has 6 kelch motifs in the C-terminal half, which are known to be a novel type of actin-binding domain (15). We further examined colocalization of EP3α isoform with full-length, N-terminal-half or C-terminal-half muskelin by fluorescence microscopy. Full-length and C-terminal-half muskelins were colocalized with EP3α isoform at the plasma membrane, but N-terminal-half muskelin was localized into nucleus (Fig. 3). Therefore, the C-terminal half including kelch motifs is responsible for the partial localization of muskelin to the plasma membrane and maybe associates with EP3α isoform. Next, we examined the effect of muskelin on Gi activity of EP3α isoform. Muskelin significantly enhanced the inhibition by EP3α isoform of the Gs-coupled EP2 receptor-induced cAMP formation (Table 1).

DISCUSSION

In this report we have identified a protein that interacts specifically with the C-terminal tail of EP3α isoform, muskelin. This is the first report of the binding protein of prostanoid receptors. Muskelin was currently identified as a novel intracellular mediator of cell adhesive and cytoskeletal responses to thrombospondin-1 (TSP-1), a regulated macromolecular component of extracellular matrix (15). Muskelin is ubiquitously expressed in various tissues, and the majority of muskelin was reported to be detected in the cytosol and a small portion was membrane associated (15), this localization profile being consistent with the subcellular localization observed here. Although muskelin was

TABLE 1

Effects of Muskelin on the Gi Activity and Internalization of EP3α Isoform

Transfection	Gi activity (cAMP content) (%)	Internalization (specific [^{3}H]PGE_2 bound) (%)
EP3α	86.4 ± 5.7	89.3 ± 8.3
EP3α + muskelin	68.2 ± 4.5	18.7 ± 6.2

Note. After HEK 293 cells had been transiently transfected with EP3α or together with muskelin, Gi activity and the internalization level of EP3α were determined as described under Materials and Methods. The values shown are the means ± SE of triplicate experiments.

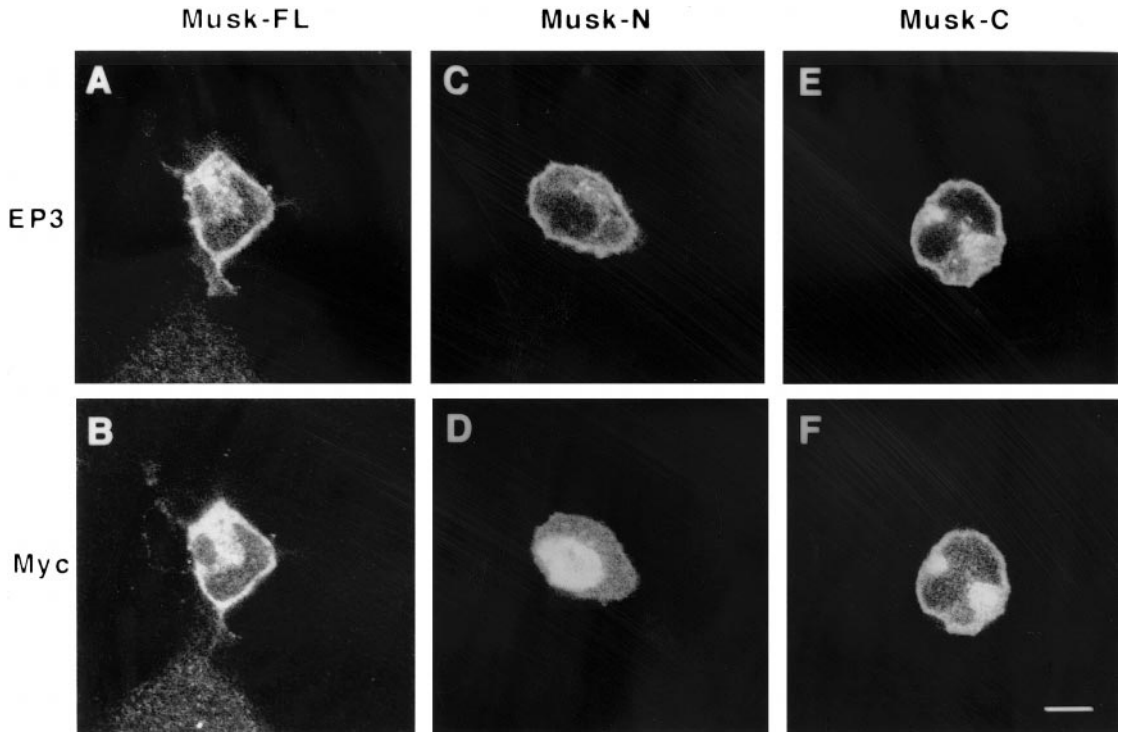

FIG. 3. Subcellular localization of full-length, N-terminal-half, and C-terminal-half muskelins in HEK 293 cells. After cells had been cotransfected with EP3α isoform and either full-length muskelin (A, B), N-terminal-half muskelin (C, D), or C-terminal-half muskelin (E, F), they were fixed and double-stained with anti-EP3 antibody (A, C, E) and 9E10 (B, D, F). Bar, 20 μm.

shown to be required for cell adhesion and spreading on TSP-1, exact molecular mechanisms for the function of muskelin within the cells has not yet been known. Previously, a multiple membrane-spanning protein, integrin-associated protein (IAP) was cloned and was revealed to be a TSP-1 receptor (16, 17). Recently, IAP was shown to associate with heterotrimeric G protein, Gi, suggesting that IAP modulates signaling pathways involving Gi (18). Considering these knowledge, it is likely that muskelin interacts with the TSP-1-IAP-Gi complex and regulates complex-mediated functions. Our results indicate that muskelin associates with EP3α isoform on the plasma membrane, suggesting that muskelin tethers the receptor to the IAP complex and modulates the receptor-Gi coupling. Actually, we showed that muskelin enhanced the Gi activity of EP3α isoform. Therefore, it is conceivable that muskelin promotes the efficiency of signal transduction from the receptor to Gi. Furthermore, we showed that EP3α isoform was internalized in response to agonist stimulation and muskelin suppressed the internalization. Thus, muskelin appears to retain EP3α isoform on the plasma membrane and insulate the receptor from internalization route, ensuring more sustained activation of the receptor.

The interaction of muskelin with EP3 receptor is specific for α isoform and muskelin does not associate with either β or γ isoform. Recently, a variety of proteins have been reported to be responsible for anchoring or subcellular targeting of G protein-coupled receptors (19–22). Most of them interact with C-terminal tails of the receptors, indicating that the C-terminal tails play important roles in the membrane targeting of the receptors. Three EP3 receptor isoforms are only different in the C-terminal tail (7, 8). Muskelin specifically recognizes the sequence of the C-terminal tail of EP3α isoform and may act as an anchor protein for the α isoform. We recently revealed that three EP3 isoforms differ in subcellular targeting (12). There may be anchor proteins specific for other two isoforms. Molecular cloning of these putative anchor proteins are now in progress in our laboratory. Different subcellular targeting of EP3 receptor isoforms would provide distinct cellular functions of the receptor dependent on targeting sites in cells. This finding will be of help in understanding the diversity of cellular responses to PGE_2.

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