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COOPERATIVE FUNCTION OF *rho* GDS AND *rho* GDI TO REGULATE *rho* p21 ACTIVATION IN SMOOTH MUSCLE[1]

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Summary: The GDP/GTP exchange reaction of *rho* p21, a member of *ras* p21-related small GTP-binding protein superfamily, is regulated by two stimulatory GDP/GTP exchange proteins (GEPs), named *smg* GDS and *rho* GDS, and by one inhibitory GEP, named *rho* GDI. In bovine aortic smooth muscle, *rho* GDS and *rho* GDI were major GEPs for *rho* p21, and the *rho* GDI activity on the GDP/GTP exchange reaction of *rho* p21 was stronger than the *rho* GDS activity in their simultaneous presence. Moreover, in the crude cytosol, the GDP-bound form of *rho* p21 was complexed with *rho* GDI but not with *rho* GDS. These results, together with our recent finding that *rho* p21 is involved in the vasoconstrictor-induced Ca^{2+} sensitization of smooth muscle contraction, suggest that there is some mechanism to release the inhibitory action of *rho* GDI and to make *rho* p21 sensitive to the stimulatory action of *rho* GDS, eventually leading to the *rho* p21 activation, in the signaling pathways of the vasoconstrictor receptors in smooth muscle.

The *rho* p21 family, consisting of three members A, B, and C, belongs to the *ras* p21-related small G protein superfamily (for reviews, see Refs. 1,2). The function of *rho* p21 has not been defined, but evidence is accumulating that *rho* p21 regulates the actomyosin system in non-muscle cells (3-5). We have previously re-

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The abbreviations used are: G protein, GTP-binding protein; GTPγS, guanosine-5'-(3-*O*-thio)-triphosphate; GEP, GDP/GTP exchange protein; EDIN, epidermal differentiation inhibitor; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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ported that *rho* p21 is a major small G protein found in smooth muscle (6) and have recently obtained the evidence that *rho* p21 is involved in the Ca^{2+} sensitization of smooth muscle contraction (7), which is induced by the addition of GTPγS to the skinned smooth muscle (8-10). It has been suggested that vasoconstrictors induce the activation of a G protein which then decreases the Ca^{2+} concentration necessary for smooth muscle contraction (8-10), but the G protein involved in this Ca^{2+} sensitization remained to be identified. Although the mode of action of *rho* p21 is still not known, it is likely that *rho* p21 affects the downstream pathway of Ca^{2+} to cause the Ca^{2+} sensitization of smooth muscle contraction.

rho p21 has two interconvertible forms: GDP-bound inactive and GTP-bound active forms (1,2,7). The GDP-bound form is converted to the GTP-bound form by a GDP/GTP exchange reaction which is regulated by GEP (1,2). There are two types of GEP: one is a stimulatory type, named *smg* GDS and *rho* GDS, and the other is an inhibitory type, named *rho* GDI (11-15). Both *smg* GDS and *rho* GDI have been well characterized. They were purified to homogeneity (11,12), their cDNAs have been cloned (14,15), and their primary structures have been determined (14,15). Both *smg* GDS and *rho* GDI are active on a group of small G proteins. *smg* GDS is active on at least Ki-*ras* p21, *smg* p21, *rho* p21, and *rac* p21 (11,16,17), whereas *rho* GDI is active on at least *rho* p21 and *rac* p21 (12,17). *rho* GDS has been just partially purified from bovine brain cytosol and less characterized (13).

The Ca^{2+} sensitization of smooth muscle contraction is observed with various vasoconstrictors such as phenylephrine and norepinephrine (8,10). Therefore, it is likely that there is some mechanism to release the inhibitory action of *rho* GDI and to make *rho* p21 sensitive to the stimulatory action of *rho* GDS, eventually leading to the *rho* p21 activation, in the signaling pathways of the vasoconstrictor receptors in smooth muscle. To clarify this regulatory mechanism of the *rho* p21 activation by these vasoconstrictor receptors, we first studied GEPs in smooth muscle. We describe here that both *rho* GDS and *rho* GDI are major GEPs in bovine aortic smooth muscle and that the *rho* p21 activity is regulated by both GEPs in a cooperative manner. The possible regulatory mechanism of the *rho* p21 activation by the vasoconstrictor receptors is also discussed.

Materials and Methods

Materials and Chemicals——Bovine aortic smooth muscle cytosol was obtained as described (18) except that a homogenizing buffer containing 20 mM Hepes/NaOH at pH 8.0 and 1 μM (*p*-amidinophenyl)methanesulfonyl fluoride was used. The post-translationally processed form of *rho*A p21 was purified from the membrane fraction of *Spodoptera frugiperda* cells (Sf9 insect cells) in which *rho*A p21 was overexpressed using a pAcYM1 *Autographa californica* baculovirus transfer vector (16). EDIN was purified from *Staphylococcus aureus* (19). An anti-rabbit *smg* GDS polyclonal antibody was made by a routine method. Other materials were obtained as described (16).

Purification of *rho* GDS from Bovine Aortic Smooth Muscle——Bovine aortic smooth muscle cytosol (805 mg of protein, 180 ml) was applied to a Q-Sepharose column (2.6 x 10 cm) equilibrated with Buffer A (20 mM Hepes/NaOH at pH 8.0 and 5 mM $MgCl_2$). After the column was washed with 180 ml of Buffer A, elution was performed with a 540-ml linear gradient of NaCl (0-0.5 M) and 135 ml of 1 M NaCl in the same buffer and fractions of 9 ml each were collected. The *rho* GDS activity appeared as a broad single peak in Fractions 72-96. The active fractions were pooled, and solid ammonium sulfate was added to this pool to give a final concentration of 40% saturation. The sample was centrifuged at 20,000 x g for 20 min. Most of the *rho* GDS activity was precipitated. This precipitate was dissolved in 20 ml of Buffer A and dialyzed against Buffer A. The dialyzed sample was used for the present study.

Assays——[^{35}S]GTPγS-binding activity of G proteins was measured in the presence of 0.5 μM Mg^{2+} as described (20) except that the incubation time was 3 h. The *rho* GDS and *smg* GDS activities to stimulate the dissociation of [^{3}H]GDP from *rho*A p21 and *smg* p21B, respectively, were measured as described (11,13). The *rho* GDI activity to inhibit the dissociation of [^{3}H]GDP from *rho*A p21 was measured as described (12). The EDIN-catalyzed ADP-ribosylation of *rho* p21 was performed as described (21).

Other Procedures——Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (22). Protein concentrations were determined with bovine serum albumin as a standard protein (23). The radioactivities of ^{3}H- and ^{32}P-labeled samples were determined using the Beckman liquid scintillation system, model LS6000IC.

Results

When bovine aortic smooth muscle cytosol was subjected to Q-Sepharose column chromatography, the *rho* GDI activity appeared as two peaks and the *rho* GDS activity appeared as a broad single peak (**Fig. 1**). The *smg* GDS activity could not be detected, but a protein recognized by an anti-*smg* GDS antibody was observed in Fractions 68-72 (data not shown). The exact reason why the *smg* GDS activity could not be detected is unclear, but might be due to the presence of interfering materials in the cytosol fraction. *rho* GDS was active on *rho*A p21 but inactive on Ha-*ras* p21 and *smg* p25A (data not shown). The Mr value of *rho* GDS was estimated to be about 50,000 from its S value (data not shown). Judging from these

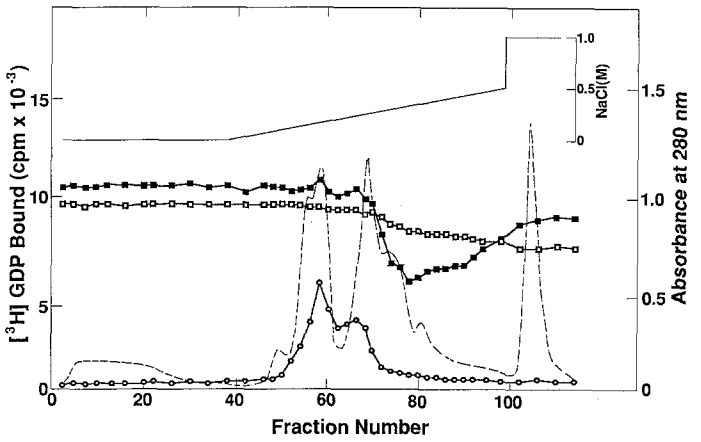

Fig. 1. Q-Sepharose column chromatography of *rho* GDI, *rho* GDS, and *smg* GDS. Aliquots (50 µl) of the indicated fractions were assayed for the *rho* GDI, *rho* GDS, and *smg* GDS activities. (○), *rho* GDI; (■), *rho* GDS; (□), *smg* GDS; (----), absorbance at 280 nm; (———), NaCl concentration. The results shown are representative of three independent experiments.

properties, *rho* GDS partially purified from bovine aortic smooth muscle cytosol was likely to be identical to that partially purified previously from bovine brain cytosol (13).

The GDP/GTP exchange reaction of *rho*A p21 is regulated by both *rho* GDS and *rho* GDI. When the GDP/GTP exchange reaction of *rho*A p21 was measured in the simultaneous presence of *rho* GDS and *rho* GDI at their doses which caused the half maximum stimulatory and inhibitory effect on the GDP/GTP exchange reaction of *rho*A p21, respectively, this reaction was inhibited by *rho* GDI even in the presence of *rho* GDS (**Fig. 2A**). The GDP/GTP exchange reaction of *rho*A p21 was not stimulated by *rho* GDS even at its higher doses in the presence of *rho* GDI (**Fig. 2B**).

When bovine aortic smooth muscle cytosol was subjected to Mono Q column chromatography, two peaks of G proteins appeared (**Fig. 3A**). The second peak (Fractions 42-48) was ADP-ribosylated by EDIN (data not shown). Among many small G proteins, only the *rho* p21 family has been shown to be selectively ADP-ribosylated by ADP-ribosyltransferases of *Clostridium botulinum* and *Staphylococcus aureus*, named C3 and EDIN, respectively (21,24-26). Moreover, we have previously found that about half of the GTPγS-binding activity of bovine aortic smooth muscle cytosol is attributed to *rho* p21 (6). These results indicate that the second peak is *rho* p21. Two peaks of *rho* GDI appeared, and the first peak of *rho* GDI coincided with the *rho* p21 peak. No small G protein was eluted at the

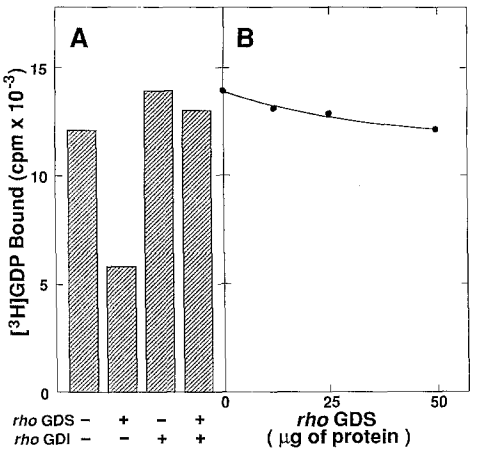

Fig. 2. Effect of *rho* GDS and *rho* GDI on the GDP/GTP exchange reaction of *rho*A p21 in their simultaneous presence. ***A,*** effect of *rho* GDS in the presence of *rho* GDI. The GDP/GTP exchange reaction of *rho*A p21 (2 pmol) was assayed by measuring the dissociation of [^{3}H]GDP in the presence of *rho* GDS (25 μg of protein), *rho* GDI (10 pmol), or both. These doses of *rho* GDS and *rho* GDI caused the half maximum stimulatory and inhibitory effect on the GDP/GTP exchange reaction of *rho*A p21, respectively. The radioactive count of [^{3}H]GDP bound to *rho*A p21 before the incubation with *rho* GDS or *rho* GDI was 15,750 cpm, and the experimental values shown were the radioactive counts retained on *rho*A p21 after the incubation. ***B,*** dose-dependent effect of *rho* GDS in the presence of *rho* GDI. The dissociation of [^{3}H]GDP from *rho*A p21 (2 pmol) was measured in the presence of *rho* GDI (10 pmol) and various doses of *rho* GDS. The results shown are the representative of three independent experiments.

position of *rho* GDS or *smg* GDS. When the cytosol was first incubated with GTPγS at a low Mg^{2+} concentration, the GDP-bound form of small G protein was mostly converted to the GTPγS-bound form. When this sample was subjected to the same Mono Q column chromatography, the first peak of *rho* GDI in **Fig. 3A** disappeared and only one peak of *rho* GDI appeared at the same position as that of the second peak of *rho* GDI in **Fig. 3A** (**Fig. 3*B***). Moreover, the second peak of G protein, which coincided with the *rho* GDI peak in **Fig. 3A**, became small. This remaining G protein might be the GDP-bound form of *rho* p21 which was not converted to the GTPγS-bound form, because this G protein was ADP-ribosylated by EDIN (data not shown). The GTPγS-bound form of *rho* p21 which became free from *rho* GDI did not appear in any fraction. This *rho* p21 might adsorb non-specifically to the matrix of the gel and/or to the column wall presumably due to its sticky property as described (16,27). In fact, when the pure sample of the GTPγS-bound form of *rho*A p21 was subjected to the same Mono Q column chromatography, it did not appear in any fraction (data not shown). However, the mixture of the GDP-bound

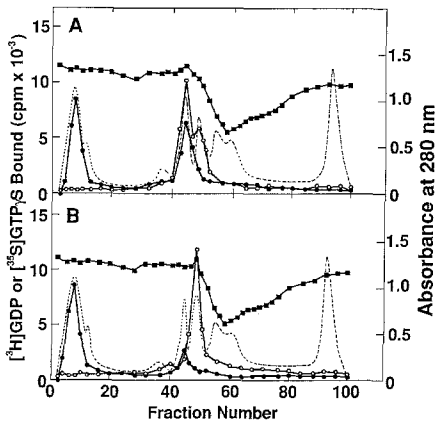

Fig. 3. Presence of *rho* p21 complexed with *rho* GDI in the cytosol of resting bovine aortic smooth muscle. Immediately after the cytosol of resting bovine aortic smooth muscle (4.7 mg of protein, 5 ml) was mixed with 10 μM GTPγS in a buffer containing 50 mM Hepes/NaOH at pH 8.0, 10 mM EDTA, and 5 mM $MgCl_2$ or after this mixture was incubated for 3 h at 30°C, 1 M $MgCl_2$ was added to give a final concentration of 20 mM to prevent the dissociation of GTPγS from G proteins. These samples were separately applied to a Mono Q column (0.5 x 5 cm) equilibrated with Buffer A. After the column was washed with 10 ml of Buffer A, elution was performed with a 30-ml linear gradient of NaCl (0-0.5 M) and 8 ml of 1 M NaCl in the same buffer. Fractions of 0.5 ml each were collected. Aliquots (50 μl) of the indicated fractions were assayed for the GTPγS-binding, *rho* GDI, and *rho* GDS activities. ***A***, without the incubation; ***B***, with the incubation. (●), G protein; (○), *rho* GDI; (■), *rho* GDS; (----), absorbance at 280 nm. The results shown are the representative of three independent experiments.

form of *rho*A p21 and *rho* GDI was subjected to the same Mono Q column chromatography, the complex appeared in the same fraction in which both *rho* p21 and *rho* GDI were eluted in **Fig. 3A** (data not shown).

Discussion

We have recently found that *rho* p21 is involved in the GTPγS-induced Ca^{2+} sensitization of smooth muscle contraction (7). This result suggests that the *rho* p21 activation is regulated by the vasoconstrictor receptors and is involved in the vasoconstrictor-induced Ca^{2+} sensitization. We have first shown here that *rho* GDS and *rho* GDI are major GEPs for *rho* p21 in bovine aortic smooth muscle. We have then shown here that the *rho* GDI activity on the GDP/GTP exchange reaction of *rho* p21 is stronger than the *rho* GDS activity in their simultaneous presence. This result is consistent with our recent observation that the *rho* GDI activity on the GDP/

GTP exchange reaction of *rho* p21 is stronger than the *smg* GDS activity in their simultaneous presence (27). Moreover, we have shown here that in resting bovine aortic smooth muscle *rho* p21 is complexed with *rho* GDI but not with *rho* GDS or *smg* GDS, and that the conversion from the GDP-bound form to the GTP-bound form makes *rho* p21 free from *rho* GDI in a cell-free system. This finding is consistent with our recent observation that in the cytosol of resting insulinoma cells *rho* p21 and *smg* p25A are complexed with *rho* GDI and *smg* p25A GDI, respectively[3]. *smg* p25A GDI is another inhibitory GDP/GTP exchange protein for a group of small G proteins including at least *smg* p25A, *rab*11 p24, and *SEC4* p24 (28-30), all of which are implicated in intracellular vesicle traffic such as exocytosis and endocytosis (1,2). On the basis of these present findings, possible modes of activation and action of *rho* p21 would be as follows: In resting smooth muscle *rho* p21 may be present in the cytosol in the GDP-bound inactive form complexed with *rho* GDI. Upon stimulation of smooth muscle with vasoconstrictors, some mechanism releases the inhibitory action of *rho* GDI and makes *rho* p21 sensitive to the stimulatory action of *rho* GDS, eventually leading to the formation of the GTP-bound active form. This GTP-bound form then decreases the Ca^{2+} concentration necessary for smooth muscle contraction. It remains to be clarified how the vasoconstrictor receptors regulate the *rho* GDI and *rho* GDS activities.

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