Interaction of recombinant rho A GTP-binding proteins with photoexcited rhodopsin

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The small molecular mass GTP-binding proteins rho A, B and C are targets for ADP-ribosyltransferase activity of the botulinum exoenzyme C3. The possible interaction of recombinant rho A proteins expressed in *E. coli* with photoexcited rhodopsin was studied by reconstitution with bovine rod outer segment (ROS) membranes depleted of endogenous GTP-binding proteins by treatment with urea. As reported for C3 substrates present in untreated ROS membranes, ADP-ribosylation of recombinant rho A proteins, both normal and Val-14 mutant, by C3 was inhibited when reconstituted with illuminated compared to dark-adapted ROS membranes pretreated with urea. GDP reduced the light-induced inhibition, while GTP[S] and light inhibited ADP-ribosylation of rho A proteins in a synergistic manner.

Rho protein; GTP-binding protein; Rhodopsin; Botulinum C3 ADP-ribosyltransferase; Bovine rod outer segment

1. INTRODUCTION

The rho proteins belong to a large family of low molecular mass (M_r's of 20–30 kDa) GTP-binding proteins (G proteins). Three members of the rho protein family, A, B and C, are known to occur in human tissues, with more than 90% sequence homology between each other [1,2]. Although the function of the rho proteins is not known, they have been suggested to be involved in regulation of cell proliferation and differentiation, possibly by affecting the cytoskeletal system [3,4]. By analogy with the related ras proteins [5], it is assumed that rho proteins are inactive in the GDP-bound state and active in the GTP-bound form. Recently, several proteins have been described, apparently specifically regulating rho protein functions, such as a GTPase-activating protein (GAP) [6], a GDP dissociation inhibitor (GDI) [7] and a GDP dissociation stimulator (GDS) [8]. In addition, all three rho proteins can be ADP-ribosylated by the botulinum exoenzyme C3 [4,9–13].

We have recently reported that bovine rod outer segment (ROS) membranes contain two substrates for the ADP-ribosyltransferase activity of C3 [14]. Most interestingly, ADP-ribosylation of these proteins was regulated by light and guanine nucleotides in a manner similar to the ADP-ribosylation of the high molecular mass, heterotrimeric G protein transducin by pertussis toxin. ADP-ribosylation of the small (22–24 kDa) G proteins by C3 and of transducin by pertussis toxin were apparently independent of each other. These data suggested that the light receptor rhodopsin not only directly interacts with transducin [15] but probably also with the small C3 substrate G proteins. Here we report on reconstitution of ROS membranes depleted of endogenous G proteins, including the C3 substrates, with recombinant human rho A proteins and show that under these conditions ADP-ribosylation of recombinant rho proteins by C3 is regulated by light and guanine nucleotides in a manner similar to endogenous C3 substrates.

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2. MATERIALS AND METHODS

2.1. *Materials*

Expression of human rho A protein and its Val-14 mutant in *E. coli* and partial purification of these recombinant proteins were as described [6]. All other materials were obtained as in [14].

2.2. *Preparation of ROS membranes*

Untreated ROS membranes were prepared in dim red light from bovine retinas according to [16]. Transducin was eluted from illuminated ROS membranes by repeated (3 ×) hypotonic elution in the presence of 100 μM GTP [14]. Urea-treated ROS membranes were prepared according to [17]. After pelleting of crude dark-adapted ROS membranes (20 mg protein) by centrifugation for 30 min at 300 000 × *g*, the pellet was washed twice with 5 ml of a buffer containing 100 mM Tris-HCl, pH 8.0, 5 M urea and 5 mM EDTA. The final pellet was resuspended in the above urea-containing buffer and incubated for 2 h at 4°C. Subsequently, the membranes were pelleted again and washed (3 ×) with 5 ml of 10 mM triethanolamine-HCl, pH 7.4, and resuspended in 1 ml of the triethanolamine buffer. Finally, the membrane suspension was dialyzed for 12 h at 4°C against the triethanolamine buffer for removing remaining urea. All procedures were performed in the dark or under dim red light.

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2.3. *ADP-ribosylation*

[^{32}P]ADP-ribosylation of ROS membrane-bound or released transducin by pertussis toxin and of endogenous small G proteins by C3 was performed as in [14]. [^{32}P]ADP-ribosylation of recombinant normal rho A and Val-14 rho A proteins (0.3 μg protein/tube each) by C3 was performed in a reaction mixture containing 50 mM triethanolamine-HCl, pH 7.4, 2 mM EDTA, 1 mM dithiothreitol, 0.5 mM ATP, 30 μM free Mg^{2+}, 0.1 μM [^{32}P]NAD (0.3–1 μCi/tube) and 0.2 μg purified C3 in a total volume of 50 μl for 15 min at 37°C. Reaction was started by addition of urea-treated ROS membranes (15 μg protein/tube) and rho proteins, pre-incubated with each other for 5 min on ice either in dim red light or in bright white light. SDS-PAGE and autoradiography were as in [14].

3. RESULTS

In contrast to transducin, the endogenous C3 substrates are not released from ROS membranes by GTP. As illustrated in Fig. 1, treatment of illuminated ROS membranes under hypotonic conditions with GTP (100 μM) decreased the amount of [^{32}P]ADP-ribose incorporated by pertussis toxin into the membranes and transducin is found in the supernatant [15]. On the other hand, under identical conditions, the amount of [^{32}P]ADP-ribose incorporated into small membrane-associated G proteins caused by C3 was not decreased and no labelling of proteins by C3 was observed in the supernatant. The increase in labelling by C3 observed in transducin-depleted membranes (lane 5) is most likely due to the fact that the assays contained the same amount of total membrane protein, and that by the elution of transducin the membranes were relatively enriched in small G proteins.

When the ROS membranes were treated with 5 M urea, as described in section 2, the membranes were not only free of transducin ([17], data not shown) but apparently also of endogenous C3 substrates. As shown in Fig. 2, no labelling of C3 substrates was detected in urea-treated ROS membranes. This may be due to release and/or denaturation of the proteins by the urea treatment. Since the urea-treated ROS membranes essentially contain only rhodopsin, but in a still activatable form [17], they were used for reconstitution with the recombinant rho A proteins. When normal A protein was added to these membranes, [^{32}]ADP-ribosylation by C3 was increased. This may be due to the presence of membrane phospholipids, which recently have been reported to increase the rate of ADP-ribosylation of bovine brain cytosolic substrates by C3 [18]. Most important, when the rho A protein was reconstituted with the urea-treated ROS membranes under bright light, labelling of the rho A protein by C3 was decreased compared to dim red light conditions.

As was reported for the endogenous C3 substrates [14], the recombinant rho A protein migrated in the SDS-PAGE as a double band, with the less intensively labelled band exhibiting a somewhat larger apparent size. Similar data have been reported for rho C protein expressed in *E. coli* and was attributed to formation of

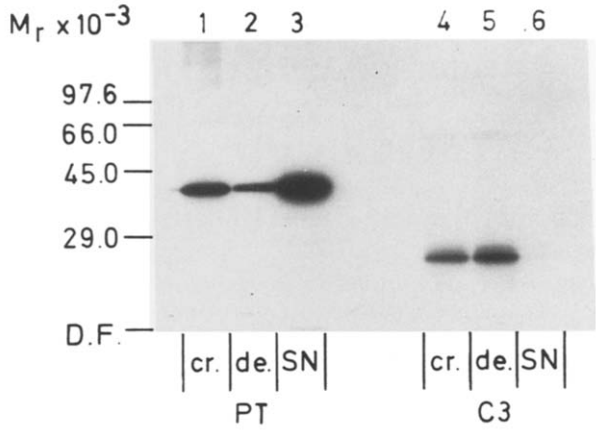

Fig. 1. ROS membrane binding of transducin and the C3 substrates. [^{32}P]ADP-ribosylation of transducin by pertussis toxin (PT, lanes 1–3) and of small G proteins by C3 (lanes 4–6) was performed as in [14] with illuminated crude ROS membranes (cr.) or membranes depleted of transducin by elution with GTP (de.) (15 μg of each membrane protein). In addition, [^{32}P]ADP-ribosylation by pertussis toxin and C3 was performed with a supernatant (SN) released by GTP from the ROS membranes. Shown is an autoradiogram with the molecular mass markers on the left. (D.F., dye front)

two conformational isomers of the same protein [4]. When reconstituted with urea-treated ROS membranes, labelling of either band by C3 was decreased by illumination (Fig. 3). In the presence of GDP (10 μM), inhibition by illumination of ADP-ribose incorporation into the recombinant rho A protein by C3 was reduced.

Not only the normal rho A protein but also its Val-14 mutant, exhibiting reduced GTPase activity [6], can be ADP-ribosylated by C3 [12]. As observed for normal rho A protein (see Fig. 2), reconstitution of Val-14 rho

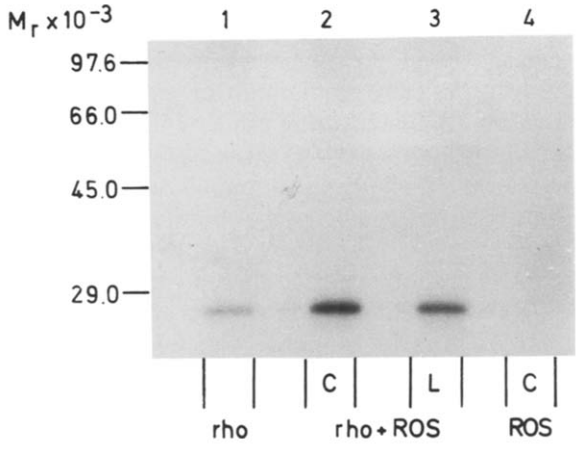

Fig. 2. Reconstitution of normal rho A protein with urea-treated ROS membranes. [^{32}P]ADP-ribosylation of partially purified normal rho A protein (0.3 μg protein) by C3 was performed in the absence of ROS membranes (lane 1) and in the presence of urea-treated ROS membranes (15 μg protein) either in dim red light (C, lane 2) or in bright white light (L, lane 3). In lane 4, urea-treated ROS membranes were incubated with C3 and [^{32}P]NAD in the absence of rho proteins. Shown is an autoradiogram.

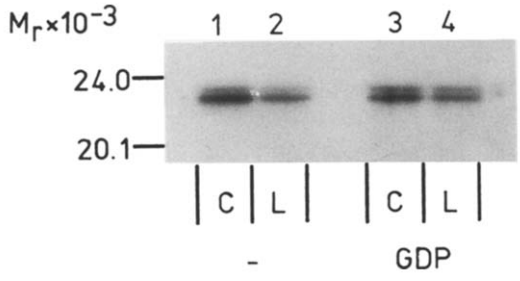

Fig. 3. Regulation of ADP-ribosylation of normal rho A protein reconstituted with urea-treated ROS membranes by light and GDP. [^{32}P]ADP-ribosylation of partially purified normal rho A protein (0.3 μg protein) by C3 was performed in the presence of dark-adapted urea-treated ROS membranes (15 μg protein) either in dim red light (C, lanes 1 and 3) or in bright white light (L, lanes 2 and 4) in the absence (-) or presence of 10 μM GDP as indicated. Shown is an autoradiogram.

A protein with dark-adapted urea-treated ROS membranes increased [^{32}P]ADP-ribosylation by C3 (data not shown). Furthermore, when the Val-14 rho A protein was combined with the ROS membranes under bright white light, [^{32}P]ADP-ribosylation by C3 was decreased compared to dim red light conditions (Fig. 4). Similar to the normal rho A protein, the presence of GDP (10 μM) inhibited or prevented the light-induced decrease in [^{32}P]ADP-ribosylation of the Val-14 rho A protein. When [^{32}P]ADP-ribosylation of Val-14 rho A protein combined with urea-treated ROS membranes was performed in the presence of the poorly hydrolyzable GTP analog, guanosine-5′-*O*-(3-thiotriphosphate) (GTP[S], 10 μM), ADP-ribosylation by C3 was decreased, even when studied under dim red light conditions. Illumination of the ROS membranes caused a further substantial decrease in [^{32}P]ADP-ribosylation.

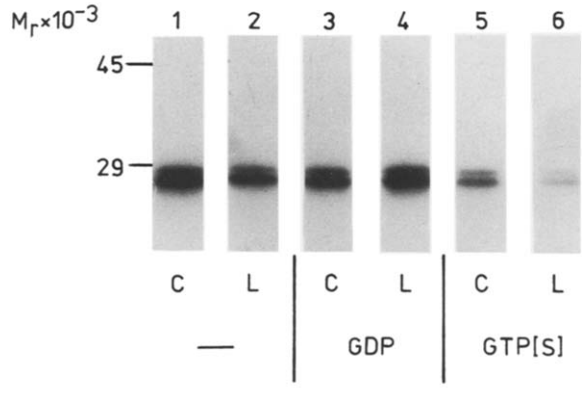

Fig. 4. Regulation of ADP-ribosylation of Val-14 rho A protein reconstituted with urea-treated ROS membranes by light and guanine nucleotides. [^{32}P]ADP-ribosylation of partially purified Val-14 rho A protein (0.3 μg protein) by C3 was performed in the presence of dark-adapted urea-treated ROS membranes (15 μg protein) either in dim red light (C, lanes 1,3,5) or in bright white light (L, lanes 2,4,6) in the absence (-) and presence of GDP (10 μM) or GTP[S] (10 μM) as indicated. Shown is an autoradiogram.

4. DISCUSSION

ADP-ribosylation of small molecular mass GTP-binding proteins present in bovine ROS membranes by the botulinum exoenzyme C3 is regulated by light and guanine nucleotides in a manner similar to the ADP-ribosylation of transducin by pertussin toxin [14]. These data suggested that these endogenous small G proteins may interact with the light receptor rhodopsin as it is known for the heterotrimeric G protein transducin [15]. In contrast to transducin, the C3 substrate G proteins are not released from illuminated membranes in the presence of GTP, which data indicate that these proteins have a more distinct membrane attachment than transducin and suggest that the possible target of these proteins is different from that of transducin.

Treatment of ROS membranes with 5 M urea causes a depletion of most of the endogenous proteins, with the exception of rhodopsin still being capable of interacting with transducin in a light-dependent manner [17]. Therefore, we used these membranes to study whether the light regulation of C3 substrates observed in crude ROS membranes is due to a direct interaction of rhodopsin with the small G proteins. Since rho proteins (A, B and C) are substrates of C3 [4,9–13], we used recombinant human rho A protein expressed in *E. coli* for the reconstitution with urea-treated ROS membranes. As is shown herein, ADP-ribosylation of rho A protein by C3 is regulated by light when studied in this reconstitution assay in a manner similar to endogenous C3 substrates. Furthermore, as has been reported for the endogenous C3 substrates [14], the presence of the nucleoside diphosphate GDP inhibited or prevented the influence caused by illumination of the ROS membranes. Furthermore, the metabolically stable GTP analog GTP[S] inhibited ADP-ribosylation of endogenous C3 substrates and of the recombinant rho A proteins. This inhibition was further increased by illumination of the ROS membranes. The endogenous C3 substrates and the recombinant rho A proteins [^{32}P]ADP-ribosylated by C3 comigrated on SDS-PAGE (data not shown). Since however rac proteins with molecular masses and amino acid compositions similar to rho proteins are also substrates for C3 [19] and since highly sensitive and specific antibodies for either of these small G proteins are not available, final elucidation of the small G protein subtype present in bovine ROS membranes and interacting with rhodopsin will require purification and amino acid analysis of the purified protein.

Interestingly, identical data were obtained with normal rho A and with Val-14 rho protein, the latter exhibiting reduced basal and rho GAP-stimulated GTPase activities [6]. These data suggest that GTP hydrolysis caused by these proteins is most likely not involved in the observed regulation by light. The regulatory effects observed with GDP and GTP[S] were

very similar when studied with the endogenous C3 substrates present in crude ROS membranes [14] and, as shown herein, with the normal and mutated Val-14 rho A proteins reconstituted with urea-treated ROS membranes. The presence of GDP inhibited the regulation by light of either protein, but GDP had essentially no effect on ADP-ribosylation performed under dim red light conditions. Therefore, it is conceivable to assume that binding of this nucleotide to the proteins prevents their interaction with photoexcited rhodopsin. On the other hand, the additive or synergistic effects observed with GTP[S] and light do not necessarily indicate that photo-excited rhodopsin stimulates binding of this nucleotide to the small G proteins as it is known for transducin [15]. Together with the GDP data, the observed additivity may even suggest that activated rhodopsin preferentially interacts with the GTP-bound configuration of the rho proteins and that inhibition of ADP-ribosylation caused by these two agents, i.e. GTP[S] and light, is due to distinct molecular mechanisms. If the rho proteins interact with photo-excited rhodopsin preferentially in their GTP-bound configuration, the intriguing possibility has to be considered that photo-excited rhodopsin is not regulating the function of these small G proteins as it does for transducin [15], but that rhodopsin is a functional target of the small G proteins, if the assumption is correct that these small G proteins are active in the GTP-bound form as are the high molecular mass heterotrimeric G proteins.

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