Binding of [α - 32 P]NTP to Cholera Toxin and Light Signal Transmission Stimulating the Binding of [α - 32 P]GTP via Pea Plasma Membrane

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Bacterial toxins such as cholera toxin (Gill and Coburn 1987), diphtheria toxin (Brown and Bodley 1979) and pertussis toxin (Katada *et al.* 1982) are well known to control signal transducing systems in animals by the ADP-ribosylation of GTP-binding proteins (Bourne *et al.* 1990). The process of penetration of cholera toxin through the plasma membrane includes the attachment of the toxin by binding of the B oligomer to the plasma membrane and then penetration of the A protomer through the plasma membrane (Gill 1976). During the penetration of the A protomer through the plasma membrane the 5 kDa A-1 part is removed by proteolysis and the A-2 part functions to catalyse ADP-ribosylation of arginine residue of target protein. The function as a toxin has been well analyzed in cholera toxin, diphtheria toxin and pertussis toxins (De Wolf *et al.* 1981). The actual function in the cell, however, is not known. Cholera toxin, diphtheria toxin and pertussis toxin ADP-ribosylate arginine, diphthamide and cysteine residue in proteins, respectively. These ADP-ribosylation were known to be activated in the presence of ATP or GTP at 10^{-4} or 10^{-3} M (Moss and Vaughan 1977). The function of these properties is unknown. Cholera toxin, diphtheria toxin and pertussis toxin with different substrate specificities are also considered to be model systems to analyze the function of endogenous ADP-ribosyl transferase (Hasunuma 1992). In the present study we found that [α - 32 P]GTP could bind with the B oligomer of cholera toxin and the mixing of plasma membrane from the red light irradiated third internode of etiolated pea seedlings with cholera toxin stimulated the binding of [α - 32 P]GTP to the toxin.

Materials and methods

Chemical. A protomer, B oligomer and whole cholera toxin were purchased from List Biol. Lab. Inc. (Campbell, CA, USA). ATP, GTP, CTP and UTP were from Pharmacia (Uppsala, Sweden). Leupeptin, pepstatin A and Tris were from Sigma Chemical Co. (St. Louis, MO, USA). PIPES and SDS were from Wako Pure Chem. Co. (Osaka, Japan). PMSF was from Boehringer Mannheim GmbH (Mannheim, FRG). Dithiothreitol and acrylamide were from Nacalai Tesque (Kyoto, Japan). The protein assay kit and prestained protein molecular weight markers were from Bio-Rad Lab. (Richmond, CA, USA). [α - 32 P]ATP (800 Ci/mmol, NEG-003X), [α - 32 P]GTP (800 Ci/mmol, NEG-006X), [α - 32 P]CTP (800 Ci/mmol, NEG-008X10), [α - 32 P]UTP (800 Ci/mmol, NEG-007X10) and [32 P]NAD (800 Ci/mmol, NEG-023X10) were from NEN Res. Prod. (Boston, MA, USA). Nitrocellulose filters (0.20 μ m) were from Schleicher & Schuell GmbH (Dassel, FRG).

Binding and UV-induced crosslinking of [α - 32 P]ATP or [α - 32 P]GTP to proteins. The standard reaction mixture (20 μ l) contained 20 mM PIPES, pH 7.0, 1.5 mM MgCl₂, 0.1 mM EDTA, 0.1 M NaCl, 0.2 mM PMSF, 0.1% Triton X-100 and 2.5 μ Ci [α - 32 P]ATP, [α - 32 P]GTP, [α - 32 P]CTP or [α - 32 P]UTP. After addition of proteins such as cholera toxin, the reaction mixture

was incubated at 25°C for 30 min. The reaction mixtures were irradiated with UV (18 W germicidal lamp; Hitachi, Hitachi, Japan) at a distance of 15 cm at 0°C for 15 min to induce crosslinking of bound nucleotides to proteins (Basu and Modak 1987). To the sample 20 μ l of 2X SDS-sample buffer containing 0.125 M Tris-HCl, pH 6.8, 10% SDS, 2 mM dithiothreitol, 10% glycerol and 0.004% bromophenol blue was added. The proteins were separated by a 5–20% SDS-polyacrylamide gel electrophoresis. Proteins in the gel were transferred by electroblotting to a nitrocellulose filter (0.20 μ m). The radioactivity on the filter was detected on Kodak X-Omat AR film directly or after staining with amido black. The molecular weight of each protein was estimated using the prestained molecular weight markers (Bio-Rad) as the standards. The molecular weights of the markers were calibrated with bovine serum albumin (67 kDa), chymotrypsinogen A (25 kDa) and cytochrome C (12.4 kDa) as the standards.

Growth of plants and purification of plasma membrane. Four hundred pea seeds were imbibed at 25°C in 1 l of Hoagland's solution in darkness for 4–5 hr and were sown in 5 l of Vermiculite moistened with 2.8 l of Hoagland's solution. From the plants grown at 25°C under complete darkness for 160 hr, the third internode was harvested under darkness using a Noctovision system (NVR 2015, Nihon Electric Co., Hamamatsu, Japan) with infra-red as a safelight, and 100 pieces per dish were kept in darkness floating on 5 ml of H₂O for 1 hr at room temperature. The sample was irradiated with red (660 nm) light for 40 sec at 0, 60, 120 and 250 μ moles/m²/sec at the sample. The following procedures were performed using the Noctovision system with an infra-red safelight, unless otherwise indicated. Stem sections were ground immediately after red light irradiation in 5 ml of a buffer containing 0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.01 mM pepstatin A, 0.01 mM leupeptin and 0.5 mM PMSF in a prechilled mortar on ice. The homogenate was squeezed through nylon cloth, then centrifuged for 10 min at 9,000 rpm (6,000 $\times$ g). The supernatant was further centrifuged at 39,000 rpm (105,000 $\times$ g) for 30 min. The resulting pellet was resuspended in 1 ml of resuspension medium (0.25 M sucrose, 5 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA, 0.01 mM pepstatin A, 0.01 mM leupeptin and 0.5 mM PMSF). It was homogenized 10 times with a Teflon homogenizer, then the plasma membrane was purified by aqueous two-phase partitioning (Widell 1987). The upper phase of the second aqueous two-phase separation was diluted with 2 volumes of resuspension medium and centrifuged at 105,000 $\times$ g for 45 min. The pellet was homogenized with a Teflon homogenizer in 1 ml of resuspension medium containing 0.1 M NaCl. The purified plasma membrane (200 μ g protein/ml) was aliquoted to 200 μ l and stored at –80°C.

Abbreviations:

PIPES, piperazine-N, N'-bis(2-ethane sulfonic acid);

SDS, sodium dodecyl sulfate;

PMSF, phenylmethylsulfonyl fluoride;

[α -³²P]ATP, [α -³²P]adenosine 5'-triphosphate;

[α -³²P]GTP, [α -³²P]guanosine 5'-triphosphate;

[α -³²P]CTP, [α -³²P]cytidine 5'-triphosphate;

[α -³²P]UTP, [α -³²P]uridine 5'-triphosphate;

Results

Binding of [α -³²P]NTP to subunits of cholera toxin. The binding of [α -³²P]ATP, [α -³²P]GTP, [α -³²P]CTP and [α -³²P]UTP at 1.6×10^{-7} M to the subunits of cholera toxin was examined by use of the affinity label method with UV irradiation induced crosslinking of tightly bound nucleotides. In the analysis, the binding of [α -³²P]NTP in the absence or presence of

29 kDa and 55 kDa proteins were detected. Proteins with 11.6 kDa, 22 kDa and 55 kDa were estimated to be monomer, dimer and pentamer of the binding subunits, and that with 29 kDa is considered to be the A protomer. The binding of [α - 32 P]ATP to the binding subunits was stimulated by ATP, GTP, CTP or UTP at a concentration of 10^{-4} M but reduced by these nucleotides at a concentration of 10^{-3} M. The binding of [α - 32 P]GTP to binding subunits in the absence of added NTP was prominent. The binding of [α - 32 P]GTP was moderately inhibited by 10^{-3} M ATP, GTP, CTP or UTP and strongly inhibited by 10^{-4} M ATP, GTP or

Fig. 2. Nucleotide binding specificity of subunits for [α - 32 P]ATP, [α - 32 P]GTP, [α - 32 P]CTP and [α - 32 P]UTP. Cholera toxin A protomer (5 μ g), B oligomer (5 μ g), a 1 to 1 (w/w) mixture of them (5 μ g each) or total enzyme (10 μ g) was included in each reaction mixture which contained 1.6×10^{-7} M [α - 32 P]ATP, [α - 32 P]GTP, [α - 32 P]CTP or [α - 32 P]UTP. The reaction mixture was incubated at 25°C for 30 min and the following procedures were as described in Materials and Methods, except that after addition of 2X SDS sample buffer, the reaction mixture was heated for 5 min at 98°C. Symbols were the same as those described in Fig. 1.

UTP. The binding of [α - 32 P]CTP to the binding subunits and to the A protomer was relatively strong, and was hardly inhibited by CTP at a concentration of 10^{-3} M. The binding of [α - 32 P]CTP was moderately inhibited by ATP, GTP or UTP at a concentration of 10^{-4} M. The binding of [α - 32 P]UTP to the binding subunits and the A protomer was relatively strong but was strongly inhibited by ATP, GTP, CTP or UTP at a concentration of 10^{-4} or 10^{-3} M.

In the former experiments the binding capacity of the subunits for [α - 32 P]NTP was examined by using total cholera toxin consisting of the A protomer and B oligomer. In this experiment the purified A protomer, purified B oligomer, 1 to 1 mixture of them, and total cholera toxin was used and after UV crosslinking of bound nucleotides, the reaction mixture

was heated to promote the separation of B oligomer (Fig. 2). The A protomer could slightly bind $[\alpha\text{-}^{32}\text{P}]\text{GTP}$, $[\alpha\text{-}^{32}\text{P}]\text{CTP}$ and $[\alpha\text{-}^{32}\text{P}]\text{UTP}$. The B oligomer strongly bound $[\alpha\text{-}^{32}\text{P}]\text{GTP}$, less strongly bound $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ and $[\alpha\text{-}^{32}\text{P}]\text{CTP}$ than $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ and weakly bound $[\alpha\text{-}^{32}\text{P}]\text{UTP}$. The mixture of A protomer and B oligomer showed on apparent increase in the binding capacity for $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ and $[\alpha\text{-}^{32}\text{P}]\text{UTP}$. In the mixture, the $[\alpha\text{-}^{32}\text{P}]\text{NTP}$ binding characteristics remained unchanged. The binding of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ to total cholera toxin was shown to be much greater and in the binding of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, $[\alpha\text{-}^{32}\text{P}]\text{CTP}$ and $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ slightly increased. As a result of the heating treatment, the B oligomer band with a molecular weight of 55 kDa disappeared.

Fig. 3. Auto-ADP-ribosylation of cholera toxin and $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ and $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ binding in the presence of various concentrations NAD. The $[\text{P}^{32}]\text{ADP}$ -ribosylation of proteins was examined in the reaction mixture containing 1.6×10^{-7} M $[\text{P}^{32}]\text{NAD}$, in the absence or presence of 10^{-4} or 10^{-3} M ATP, GTP, CTP or UTP, and 10 μg of cholera toxin. The binding of 1.6×10^{-7} M $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ and $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ was analyzed in the absence or presence of 10^{-5} , 10^{-4} or 10^{-3} M NAD.

Symbols are the same as those described in Fig. 1.

$[\text{P}^{32}]\text{ADP}$ -ribosylation of cholera toxin showed a very complicated pattern (Fig. 3). The binding subunit might be slightly $[\text{P}^{32}]\text{ADP}$ -ribosylated as indicated from the 11.6 kDa protein, which was not affected by the presence of 10^{-4} to 10^{-3} M ATP, GTP, CTP or UTP. The radioactive proteins with molecular weights of 22 kDa and 57 kDa might be a dimer and pentamer of the 11.6 kDa protein but this was not confirmed in the present experiment. The 29 kDa protein might be the A protomer and the 24 kDa protein might correspond to the A-2 part of the A protomer, but this was not confirmed. The radioactive protein bands showing 19 kDa and 44 kDa can not be explained in the present study.

The binding of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ and $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ to the binding subunits was stimulated by NAD at a concentration of 10^{-4} M, but inhibited by that at 10^{-3} M.

Table 1 shows the schematic representation of the binding of $[\alpha\text{-}^{32}\text{P}]\text{NTP}$ to the subunits and possible ADP-ribosylation of the subunits of cholera toxin.

Light signal transmission from pea plasma membrane. The binding of GTP to the binding oligomer may be a process stimulating the function of the A protomer. Plasma membrane preparations purified from the red light irradiated (0, 60, 120 and 250 $\mu\text{moles/m}^2/\text{sec}$, for 40 sec) third internode of etiolated pea seedling were mixed with cholera toxin under infra-red light using a Noctovision system. The results in Fig. 5, clearly showed that the red light signal is transmitted to the binding of [$\alpha\text{-}^{32}\text{P}$]GTP to the binding subunits of cholera toxin. Increasing fluence rates of red light from 0 to 120 $\mu\text{moles/m}^2/\text{sec}$ stimulated the binding of [$\alpha\text{-}^{32}\text{P}$]GTP,

Table 1. [$\alpha\text{-}^{32}\text{P}$]NTP-binding and auto-[^{32}P]ADP-ribosylation of cholera toxin
A protomer and B oligomer

Subunit	Molecular weight (kDa)	Auto-[³² P]ADP ribosylation	[α- ³² P]NTP-binding			
			[α- ³² P]ATP,	-GTP,	-CTP,	-UTP
A-protomer						
A1	29	‡‡‡	—	—	—	—
A2	24	‡‡‡	—	—	—	—
B-oligomer						
Monomer	11.6	±	‡‡	‡‡‡	‡‡‡	‡‡
Pentamer	55	±	±	‡‡‡	+	+

Fig. 4. Stimulation of [$\alpha\text{-}^{32}\text{P}$]GTP binding to binding subunits of cholera toxin. Purified plasma membranes from red irradiated (40 sec at 0 (CD), 60 (R/4), 120 (R/2) and 250 $\mu\text{moles/m}^2/\text{sec}$ (R)) third internodes, were mixed with [$\alpha\text{-}^{32}\text{P}$]ATP, [$\alpha\text{-}^{32}\text{P}$]GTP, [$\alpha\text{-}^{32}\text{P}$]CTP and [$\alpha\text{-}^{32}\text{P}$]UTP. The reaction mixtures were incubated for 5 min at 0°C and the bound nucleotide was crosslinked with UV and the reaction was analyzed as described in Materials and methods. Symbols are the same as those described in Fig. 1.

but 250 $\mu\text{moles}/\text{m}^2/\text{sec}$ red light rather reduced the binding of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ to the B oligomer of cholera toxin.

Discussion

The function of the cholera toxin B oligomer has been established to be the binding of the toxin to a ganglioside on the plasma membrane in animal cells (De Wolf 1981) to support the penetration of the A protomer. ADP-ribosyl transferase activity of cholera toxin can be stimulated in the presence of 10^{-3} M GTP (Gill and Coburn 1987). The molecular basis of the activation of ADP-ribosyl transferase remains unknown. In the present work the selective binding of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ to the B oligomer was demonstrated. The binding of GTP to the B oligomer might stimulate its binding to the plasma membrane, or stimulate the release of the A protomer from the B oligomer. Since the B oligomer is known to inhibit ADP-ribosyl transferase, the binding of GTP to the B oligomer stimulating the separation of the A protomer from the B oligomer will result in the activation of ADP-ribosyl transferase. Such possibilities should be analyzed in further research.

Red light signal transmission from the plasma membrane purified from the third internodes irradiated with an increasing fluence rates of red light to B oligomer is a very intriguing phenomenon. The activation of endogenous ADP-ribosyl transferase by light signal was reported in the microsomal fraction of mycelia of *Neurospora crassa* (Oda and Hasunuma 1992), in the chloroplast of *Pisum sativum* (Millner and Robison 1989) and etiolated seedlings of *Avena sativa* (Romero *et al.* 1991). The molecular basis of this phenomenon has yet to be analyzed. The present findings suggest that endogenous ADP-ribosyl transferase is activated by the binding of NTP. Further work is needed to support this hypothesis.

Summary

Cholera toxin is composed of a catalytic subunit (29 kDa) called the A protomer and binding subunits (pentamer of 11.6 kDa subunit) called B oligomer. The binding of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, $[\alpha\text{-}^{32}\text{P}]\text{GTP}$, $[\alpha\text{-}^{32}\text{P}]\text{CTP}$ and $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ at 1.6×10^{-7} M was detected in the binding subunits, in which the binding of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ was most efficient. The binding of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ to the binding subunits was stimulated in the presence of 10^{-4} M ATP, GTP, CTP or UTP. The binding of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ was strongly inhibited in the presence of 10^{-3} M ATP, GTP or UTP. Similar results were observed in the binding of $[\alpha\text{-}^{32}\text{P}]\text{CTP}$. The binding of $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ was strongly inhibited in the presence of 10^{-4} M ATP, GTP, CTP or UTP. The binding of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ to the B oligomer was stimulated in the presence of the A protomer. The $[\text{}^{32}\text{P}]\text{ADP-ribosylation}$ of the A protomer (29 kDa) and A-2 protomer (24 kDa) lacking A-1 (5 kDa) was unaffected by ATP, GTP, CTP or UTP at a concentration of 10^{-4} or 10^{-3} M. At a concentration of 10^{-4} M NAD stimulated the binding of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ and $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ but at a concentration of 10^{-3} M it inhibited it.

The plasma membrane was purified from the red light irradiated third internode of etiolated pea seedlings. The purified plasma membrane was mixed with cholera toxin in the presence of 1.6×10^{-7} M $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, $[\alpha\text{-}^{32}\text{P}]\text{GTP}$, $[\alpha\text{-}^{32}\text{P}]\text{CTP}$ or $[\alpha\text{-}^{32}\text{P}]\text{UTP}$. The binding of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ was stimulated partly proportional to the fluence rates of red light irradiation of the third internodes.

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