A Role of the B-Oligomer Moiety of Islet-activating Protein, Pertussis Toxin, in Development of the Biological Effects on Intact Cells

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Islet-activating protein (IAP), pertussis toxin, is an oligomeric protein (Tamura, M., Nogimori, K., Murai, S., Yajima, M., Ito, K., Katada, T., Ut, M., and Ishii, S. (1982) Biochemistry 21, 5516–5522), the biggest subunit (M_r = 28,000, referred to as the A-protomer) of which catalyzes transfer of the ADP-ribose moiety of NAD to the membrane M_r = 41,000 protein. The pentamer, termed the B-oligomer, consisting of the residual subunits was the moiety of IAP that was responsible for binding to the cell surface, as revealed by competitive inhibition of the development of the IAP actions on intact rat C6 glioma cells and rat adipocytes. The binding of the B-oligomer to its receptor proteins was divalent via the constituent two dimers; it stimulated mitosis of lymphocytes and caused an insulin-like action to enhance glucose oxidation in adipocytes, just as did concanavalin A, presumably as a result of crosslinking or aggregation of the membrane proteins. The A-promoter displayed its biological action on adipocytes only when the B-oligomer had been bound to the cells. Thus, IAP is a typical A-B toxin in which the B-oligomer is first bound to the cell surface proteins to enable the A-protomer to reach to the site of its action within the cell. Diverse biological actions of pertussis toxin may be accounted for by the mitogenic action of the B-oligomer as well as ADP-ribosyltransferase activity of the A-protomer.

Islet-activating protein, pertussis toxin, exerts its unique influence on the membrane receptor-adenylate cyclase system in a variety of cell types. IAP catalyzes transfer of the ADP-ribose moiety of the intracellular NAD to an M_r = 41,000 protein of cell membranes (1–5), thereby reversing receptor-mediated or GTP-dependent inhibition of adenylate cyclase (3–5). Conceivably, the M_r = 41,000 protein is one of the subunits of the guanine nucleotide regulatory protein (N_r) that transduces a signal of receptor stimulation to the cyclase catalytic component in an inhibitory fashion; ADP-ribosylation of this protein by IAP results in loss of the Ni function (3,4). Exposure of some cell types to IAP enhanced receptor-mediated or GTP-dependent activation of membrane adenylate cyclase as well as receptor-mediated accumulation of intracellular cAMP (6–10). It has recently been argued that this enhancement also emerges from IAP-induced impairment of the capability of N_r to attenuate activation of adenylate cyclase (4).

IAP is an oligomeric protein consisting of six subunits, five of which are dissimilar to each other (11). These subunits were first named S-1 (M_r = 28,000), S-2 (23,000), S-3 (22,000), S-4 (11,700), and S-5 (9,300) according to the order of their molecular weights (11). IAP was readily dissociated to S-1 and a pentamer, an association product of the other subunits, in a concentrated urea solution (11). The S-1 was referred to as an (Active)-protomer, since it displayed ADP-ribosyltransferase or NAD-glycohydrolase activity in vitro (11,12). The pentamer then should be a (Binding)-oligomer if IAP is really one of the A-B toxins (13) as has previously been proposed (11). The purpose of the present paper is to show that IAP is bound to the cell surface at certain sites on its B-oligomer moiety to enable the A-protomer to reach the site of its action inside the cell membrane, thereby affording experimental supports to our previous proposal (11).

EXPERIMENTAL PROCEDURES

Preparation of IAP, Its Constituent Peptides, and Their Antibodies—IAP was purified from the 2-day culture supernatant of Bordetella pertussis (Tohama strain, Phase I) according to the procedure described elsewhere (14, 15). The purified IAP was dissociated into its constituent peptides, i.e., the A-protomer, Dimer-1 (S-2 plus S-4), Dimer-2 (S-3 plus S-4), and C-subunit (S-5) by being exposed to 5 M urea for 4 days at 4°C; these peptides were separated from each other by means of a column of carboxymethyl-Sepharose CL-6B, as reported in detail previously (11). The B-oligomer was prepared by combining Dimer-1, Dimer-2, and the C-subunit at the 1:1:1 molar ratio in 2 M urea. Alternatively, the B-oligomer was separated as such from the A-protomer, when the exposure time of IAP to 5 M urea was shortened to 5 h. Separation of these two fragments was readily undertaken by using haptoglobin-Sepharose 4B as an affinity column for the B-oligomer (11). Unless otherwise specified, these IAP subunits as well as IAP itself were stored in a vehicle consisting of 2 M urea and 0.1 M potassium phosphate buffer (pH 7.0) at 4°C until use. The vehicle containing no peptide was used for control experiments.

Anti-A-protomer or anti-B-oligomer rabbit antiserum was prepared by essentially the same procedure as that for anti-IAP antisera (14, 15). The IgG fraction was isolated from these antisera by affinity column chromatography using protein A-Sepharose (Pharmacia Fine Chemicals). The Ouchterlony double immunodiffusion reaction was carried out with these antibodies on 0.6% agar in 0.1 M Veronal buffer (pH 8.6) for 12 h at 37°C. The crude membrane fraction prepared from these cells (2,10) was suspended in 25 mM Tris-HCl (pH 7.5) containing 2.5 mM MgCl_2 at a concentration of 5 mg of protein/ml and stored in liquid nitrogen until use. Protein was determined by the method of Lowry et al. (17) using bovine serum albumin as standard.
The reaction mixture for ADP-ribosylation of membranes (120-160 µg of protein) was 50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 1 mM ATP, 1 mM dithiothreitol, 20 mM thymidine, 10 µM [α-32P]NAD (5 Ci/mmol), and 50 µg/ml of IAP. After a 10-min incubation at 37 °C, the membranes were washed, dissolved, and submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described elsewhere (1, 2). The radioactive band of the dried gels was excised and counted for its 32P content.

Mitogenic Activity—Splenic cells obtained from the male ICR-JCL mouse were suspended in RPMI 1640 culture medium containing 10% fetal calf serum and 5 mM HEPES at the density of 2 × 10^6 cells/ml. The cell suspension was cultured with additions of multiwell plates (Linbro) at 37 °C for 24–96 h under a humidified atmosphere of 5% CO2/96% air. [3H]Thymidine (0.2 µCi) was added to each well 24 h before the end of the culture. The cells were then collected on a glass fiber filter paper (Whatman, GF/A), washed with saline, 5% trichloroacetic acid, and methanol, and dried to be counted for radioactivity.

Glycerol Release and Glucose Oxidation in Rat Adipocytes—Rat adipocytes (1–10 × 10^6 cells) prepared by the collagenase digestion method of Rodbell (19) were suspended in 1 ml of Krebs-Bunge bicarbonate solution containing 2% bovine serum albumin. This cell suspension was incubated at 37 °C under an atmosphere of 95% O2/5% CO2 for either prior treatment with the B-oligomer or its constituent peptides or estimation of glycerol production or glucose oxidation in the presence or absence of IAP or other additions. Where indicated, preincubation was carried out at 27 °C. The pretreatment was followed by twice washing with the fresh medium containing no addition before the further incubation for estimation of the metabolic activities. Glycerol in the medium was determined enzymatically (20) after deproteinization by the Biogel-H2-ZnSO4 method. In the case of glucose oxidation assay, the incubation medium was supplemented with 0.2 mM [1-14C]glucose (0.025 µCi/ml) and 14CO2 production was measured after deproteinization with 0.2 ml of 2 M perchloric acid (19).

Materials—[1-14C]Glucose (0.25 Ci/mmol) and [6-3H]thymidine (15 Ci/mmol) were obtained from New England Nuclear. Concanavalin A and enzymes and coenzymes for glycerol assay were purchased from Sigma. The sources of all other materials used are those described in the previous papers (1–12).

Presentation of Data—In the present communication, most of the data are shown as ranges of values dependent on concentrations of additions such as IAP, its constituent peptides, or antibodies. These experiments were repeated two or three times to determine the appropriate concentration ranges, and the representative results have been presented as figures.

RESULTS

Competitive Inhibition by B-Oligomer of the Actions of Native IAP on Intact Rat C6 Glioma Cells—Membranes prepared from rat C6 glioma cells that had been exposed to 100 ng/ml of IAP for 3 h exhibited GTP-dependent adenylate cyclase activity much higher than the activity of the membranes from nontreated cells (the left end of plots in Fig. 1A; see Ref. 10). Replacement of IAP by its B-oligomer, just as was the case with aprotinin, inhibited ARP-dependent adenylate cyclase activity (open circles in Fig. 1A). The B-oligomer added 15 min prior to the IAP addition, however, inhibited the action of IAP on intact cells to enhance GTP-dependent adenylate cyclase in membranes prepared therefrom (solid circles in Fig. 1A). In contrast, the action of IAP was not affected by the B-oligomer which was added at 20 min (or later) after the onset of the exposure of intact cells to IAP (data not shown).

The action of IAP on intact C6 cells was due to ADP-ribosylation of the membrane Mv = 41,000 protein with intracellular NAD as substrate (1, 2). The fraction of the membrane proteins that remained to be ADP-ribosylated in intact cells was then radiolabeled with [32P]NAD during the subsequent incubation of the membranes prepared therefrom again with IAP (2) or its a-protomer (12). Thus, the plot of membrane GTP-dependent adenylate cyclase activities as a function of concentrations of IAP during incubation of the membrane-donor cells was a mirror image of the plot of the 32P-content of the Mv = 41,000 protein after radiolabeling of the same membranes (Fig. 1B). The increasing [32P]ADP-ribosylation in membranes reflects the ADP-ribosylation that has occurred in intact cells, making the Mv = 41,000 substrate unavailable to the subsequent reaction. The combined addition of the B-oligomer with IAP during cell incubation caused the same degree of shift of both plots to the right, reflecting the competitive inhibition of both IAP actions on intact cells by the B-oligomer (Fig. 1B).

The 32P-content of the Mv = 41,000 protein was inversely correlated with GTP-dependent adenylate cyclase activity of the same membranes (r = −0.986; p < 0.001) regardless of whether membrane-donor cells had been exposed to IAP in the presence or absence of its B-oligomer (Fig. 2). Thus, the ADP-ribosylation of the Mv = 41,000 protein is responsible for IAP-induced enhancement of GTP-dependent adenylate cyclase even in the presence of its B-oligomer. It is very likely that IAP is bound to the cell surface of intact C6 cells via particular sites on its B-oligomer moiety before the onset of its action to catalyze ADP-ribosylation of a membrane protein.

Failure of the Anti-B-oligomer Antibody to Suppress the Action of IAP on Isolated Membranes—Antisera were raised in rabbits against native IAP, its a-protomer and B-oligomer. The double immunodiffusion technique using the IgG fractions prepared from these antisera showed that the anti-IAP antibody precipitated with IAP or its constituent peptides, a-protomer and B-oligomer (Fig. 3). In the case of the anti-A-protomer or anti-B-oligomer antibody, there was a sharp precipitation arc with its respective antigen similarly whether the antigen was present in an isolated state or in an associa-
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The ADP-ribosylation data in Fig. 1B are plotted against the adenylate cyclase data obtained with the same membranes. C, without B-oligomer; ○, with B-oligomer.

**Fig. 2.** Correlation of adenylate cyclase activity with ADP-ribosylation of the $M_r = 41,000$ protein in membranes of cells treated with IAP in the presence or absence of B-oligomer. The ADP-ribosylation data in Fig. 1B are plotted against the adenylate cyclase data obtained with the same membranes. C, without B-oligomer; ○, with B-oligomer.

**Fig. 3.** Specific immunoprecipitation of IAP, its A-protomer and B-oligomer with their respective antibodies. Double immunodiffusion was carried out with 180 $\mu$g of anti-IAP (left), anti-A-protomer (center) or anti-B-oligomer (right) IgG in each center well as described under "Experimental Procedures." The contents of surrounding wells: IAP, 6 $\mu$g; A, 3.2 $\mu$g of A-protomer; B, 5.6 $\mu$g of B-oligomer.

**Fig. 4.** Suppression of the IAP actions on intact cells and isolated membranes by the antibody against its A-protomer or B-oligomer. In A, anti-A-protomer (○) or anti-B-oligomer (●) IgG was added to C6 cell cultures 15 min before the start of the usual 3-h IAP treatment. Temperature of culture was lowered to 27 °C during 15-min exposure to IgG only. GTP-dependent adenylate cyclase activity of membranes prepared from these cells is plotted against concentrations of IgG added. In B, membranes prepared from nontreated cells were submitted to the usual ADP-ribosylation reaction with IAP in the presence of anti-A-protomer (○) or anti-B-oligomer (●) IgG. ADP-ribosylation of the membrane $M_r = 41,000$ protein is plotted against concentrations of IgG used.

**Fig. 5.** Mitogenic action of IAP and its B-oligomer on mouse lymphocytes in comparison with concanavalin A. Mouse splenic lymphocytes were cultured with IAP (○), A-protomer (●), B-oligomer (●), or concanavalin A (×) at concentrations indicated on abscissa to estimate their mitogenic activities by $[^{3}H]$thymidine incorporation as described under "Experimental Procedures." Culture time is shown in each panel.

Effect of the B-oligomer of IAP in promoting DNA synthesis, whereas the A-protomer was without effect.

We have reported (11) that the B-oligomer is a pentamer in which two dimers, referred to as Dimer-1 and Dimer-2, are associated by means of a connecting subunit (C-subunit or S-5). None of these constituent peptides, Dimer-1, Dimer-2, and C-subunit, exhibited mitogenic activity when added to splenic cells each by itself (Fig. 6), though both dimers antagonized, but C-subunit did not, the action of the B-oligomer (data not shown, but see Fig. 7 later). Thus, a multiple attachment of the B-oligomer to the cell surface via its different constituent peptides, probably Dimer-1 and Dimer-2, appears to be essential for the B-oligomer (or IAP) to exhibit its mitogenic activity.

Interference with IAP-induced Glycerol Release from Rat Islet-activating Protein—The incorporation of $[^{3}H]$thymidine into DNA of mouse splenic cells was accelerated by IAP as well as by concanavalin A (Fig. 5). The strongest action was observed when cells were exposed to these proteins at concentrations of 1–2 $\mu$g/10$^6$ cells (or 2–4 $\mu$g/ml) for 48 h. IAP resembles the lectin in that it is also a T-cell mitogen; it was not effective on splenic cells prepared from a thymus-deficient nude mouse. Therefore, the B-oligomer of IAP was as effective as the native IAP in promoting DNA synthesis, whereas the A-protomer was without effect.

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Interference with IAP-induced Glycerol Release from Rat

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2 Y. Tsuchiya, K. Nogimori, K. Hosoda, and M. Ui, unpublished observations.
molar basis. IAP and its B-oligomer also mimicked the action of insulin to stimulate glucose oxidation; they were roughly equipotent to, but less efficient than, concanavalin A. More glucose was oxidized by the B-oligomer than by the native IAP at their maximal concentrations employed (Fig. 8).

**The B-Oligomer Confers the A-Protomer Responsiveness on Intact Cells**—The A-protomer of IAP was without effect on intact C6 cells, despite its capability to catalyze ADP-riboseylation of the membrane protein and to enhance GTP-dependent adenylate cyclase when added directly to the cell-free membrane preparation (12). In order to afford evidence for the indispensable role of the B-oligomer in occurrence of the A-protomer-catalyzed reactions in intact cells, we used the B-oligomer-bound adipocytes, which were prepared as in Fig. 7, again in experiments shown in Fig. 9.

In accord with our previous observation with C6 cells (12), the A-protomer of IAP failed to stimulate glycerol release during incubation of the fresh fat cells. When fat cells bound with the B-oligomer were incubated, however, the addition of the A-protomer increased glycerol release therefrom in a concentration-dependent manner (Fig. 9). It is conceivable that the A-protomer was allowed to gain access to the site of

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**Table I**

**Specific inhibition of IAP-induced glycerol release from adipocytes by B-oligomer**

| Preincubated with B-oligomer | Glycerol release during incubation with | Glycogen
<table>
<thead>
<tr>
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<tr>
<td>None</td>
<td>IAP</td>
</tr>
<tr>
<td></td>
<td>Cholera toxin</td>
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<tr>
<td></td>
<td>Glucagon</td>
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<tr>
<td>&lt;sup&gt;−&lt;/sup&gt;</td>
<td>0.20 ± 0.03</td>
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<td>2.81 ± 0.15</td>
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<td>1.90 ± 0.10</td>
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<td>1.02 ± 0.05</td>
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<td></td>
<td>1.11 ± 0.05</td>
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<td>+</td>
<td>0.25 ± 0.04</td>
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<td>0.30 ± 0.03</td>
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<td>1.82 ± 0.13</td>
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<td>1.37 ± 0.13</td>
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*Effect of B-oligomer pretreatment is significant (p < 0.001).*

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**Fig. 7. Inhibition of IAP-induced glycerol release from rat adipocytes by B-oligomer or its constituent peptides.** In A, adipocytes (4 × 10<sup>6</sup> cells/ml) were preincubated without (C) or with 0.3 (D), 1 (A), or 5 (E) μg/ml of B-oligomer for 5 min at 37 °C before 3-h incubation at the same cell density with various concentrations of IAP as described under "Experimental Procedures." In B, adipocytes (2 × 10<sup>5</sup> cells/ml) were preincubated with various concentrations of B-oligomer (D), Dimer-1 (A), Dimer-2 (V), or C-subunit (H) for 10 min at 27 °C before 210-min incubation with 30 ng/ml of IAP. Glycerol released during incubation with IAP is expressed as a percentage of the control value (obtained with adipocytes preincubated with vehicle only) in B.
Role of B-Oligomer of Islet-activating Protein

The biggest subunit (S-1), referred to as the A-protomer, of IAP has been found to be an enzyme that catalyzes transfer of the ADP-ribosyl moiety of NAD to a membrane protein of rat C6 glioma cells (11, 12). The unique action of IAP on various cell types is reasonably accounted for in terms of this enzymic activity (4), despite failure of the A-protomer by itself to act on intact cells (12). Thus, the oligomeric protein consisting of the residual subunits of IAP was termed the B-oligomer with the expectation that it would bind to particular sites on the cell surface thereby rendering the associated A-protomer to traverse the plasma membrane to reach to the site of its action within the cell (11). The experimental data presented above are in accordance with this expectation; the B-oligomer competed with IAP for the same site(s) on C6 cells (Fig. 1). The biological action of IAP on intact adipocytes was also inhibited competitively by the B-oligomer (Fig. 7). The B-oligomer was not inhibitory in either cell type unless it was added before the onset of IAP action. The indispensable role of the B-oligomer moiety in the cellular action of IAP was verified by the inhibition of the action of IAP on intact C6 cells by the anti-B-oligomer antibody which was ineffective on the action of IAP on the cell-free membranes (Fig. 4).

Radioassay for binding of IAP or its B-oligomer to cells was not feasible because the subunit assembly of these proteins was readily impaired by iodination of their constituent amino acids. Instead, mitosis of lymphocytes and glucose oxidation in adipocytes have been employed in the present study as alternative indices of protein binding to the cell surface. It is well known that a lectin such as concanavalin A exhibits mitogenic activity as a result of its multivalent attachment to glycopolysaccharides on the cell surface; cross-linking or clustering of these receptor proteins is probably involved in mitogenicity (24–27). Thus, increased [3H]thymidine incorporation into splenic cells caused by the B-oligomer of IAP may reflect its multivalent binding to membrane proteins. The subunit structure of the B-oligomer (11) is such that it consists of two dimers (Dimer-1 and Dimer-2) which are connected by the C-subunit (S-5). It is very likely that the B-oligomer is bound divalent to the surface of the cell by means of the two dimers, since any of the dimers was not mitogenic by itself (Fig. 6) despite their binding to the cells as revealed by their competition with IAP on adipocytes (Fig. 7). The C-subunit does not appear to possess a binding site; it was without effect on the biological activity of IAP in adipocytes (Fig. 7). IAP must be bound to the cell surface in the same manner as its B-oligomer, because both proteins were equally effective as mitogens (Fig. 5). The B-oligomer is the moiety that is responsible for the binding of IAP to the cell membrane.

The insulin-like activity of IAP and its B-oligomer to enhance glucose oxidation in adipocytes (Fig. 8) affords additional evidence for divalent binding of the B-oligomer to the cell surface. Cross-linking or aggregation of receptor proteins achieved by multilinkpoint attachment to the proteins has been suggested to be responsible for the insulin-like action of the antibodies to the insulin receptors (28) or intrinsic membrane proteins (29) as well as concanavalin A (30, 31). The same mechanism must be involved in the insulin-like action of the B-oligomer of IAP. The native IAP was less effective than its B-oligomer in stimulating glucose oxidation in adipocytes. Presumably, the insulin-like action displayed by the B-oligomer moiety of IAP attached to the fat cell surface was partially cancelled by the subsequent entry of its A-protomer moiety which results in tremendous increases in the cellular cAMP content. In accord with this notion, glucagon or epinephrine, which acts on adipocytes via generation of cAMP, is somewhat antagonistic to insulin, e.g. lipolysis is increased by glucagon or epinephrine while decreased by insulin.

Pertussis toxin exhibits such diverse biological activities as to be termed lymphocyte-stimulating-promoting factor, histamine-sensitizing factor, hemagglutinin, apart from IAP (32, 33). In addition, the toxin acts as a potent adjuvant. Our findings that not only the A-protomer but also the B-oligomer was biologically active when IAP interacted with various cell types will contribute to elucidation of such diversity of the toxin action; we would propose that mitogenicity of the B-oligomer shown in the present study plays an important role in development of lymphocyte-stimulating-promoting, hemagglutinating, and adjuvant activities of pertussis toxin. The in vitro mitogenicity of pertussis toxin for mouse T-lymphocytes was previously described by Kong and Morse (34, 35).

In summary, the B-oligomer is the moiety of IAP directly involved in the IAP binding to the cell surface. The binding is the first step required for the A-protomer, ADP-ribosyl-transferase, of IAP to enter the cell. Actually, the A-protomer was ineffective on adipocytes unless the B-oligomer had been bound to the cells (Fig. 9). Since the binding of the B-oligomer to the cell surface is divalent via the two constituent dimers (Dimer-1 and Dimer-2) connected with each other by means of another subunit (C-subunit), it stimulates the cell, probably as a result of cross-linking or aggregation of the binding proteins on the cell membrane, in such a fashion as to cause mitosis of lymphocytes or insulin-like action on adipocytes. Further studies are now in progress in our laboratory for a possible role of the B-oligomer of IAP in diverse biological actions of whooping cough bacteria.

REFERENCES

Role of B-Oligomer of Islet-activating Protein

A role of the B-oligomer moiety of islet-activating protein, pertussis toxin, in development of the biological effects on intact cells.

M Tamura, K Nogimori, M Yajima, K Ase and M Ui


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