Structure–Function Relationship of Islet-Activating Protein, Pertussis Toxin: Biological Activities of Hybrid Toxins Reconstituted from Native and Methylated Subunits

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ABSTRACT: Islet-activating protein (IAP), pertussis toxin, is a hexameric protein composed of an A protomer and a B oligomer, the residual pentamer having such a subunit assembly that two different dimers, dimer 1 and dimer 2, are connected with each other by means of the smallest C subunit. Incubation of IAP with formaldehyde and pyridine–borane produced the modified toxin in which most of the free amino groups were dimethylated. The methylated and nonmethylated (native) IAP were disintegrated into their respective constituent components, which were then cross combined to reconstitute hybrid toxins with the original hexameric structure. The binding of the B oligomer to the mammalian cell surface via dimer 2 was, but the binding via dimer 1 was not, seriously impaired by methylation of amino groups in the protein. The binding of the B oligomer allowed the A protomer to enter cells and to catalyze ADP-ribosylation of a membrane M_r 41 000 protein. The diverse biological activities of IAP occurring by this mechanism were mimicked by not only methylated IAP but also all hybrid toxins, indicating that the free amino groups in the protein were not essential for the enzyme activity of the A protomer and that the A protomer was able to enter cells if the B oligomer bound to cells "monovalently" via dimer 1. An additional effect of the B oligomer binding, i.e., the direct stimulation, without the transport of the A protomer, of cells leading to mitosis in lymphocytes in vitro or increases in circulating lymphocytes in vivo, was not mimicked by hybrid toxins containing methylated dimer 2, indicating that "divalent" binding via both dimers was indispensable in cell stimulation. Thus, dual mechanisms are proposed for binding of pertussis toxin to mammalian cells.

Islet-activating protein (IAP),[1] pertussis toxin, is an exotoxin produced by *Bordetella pertussis*, whooping cough bacteria (Ui, 1984). The toxin is a hexamer composed of five dissimilar subunits termed S-1 (M_r 28 000), S-2 (M_r 23 000), S-3 (M_r 22 000), S-4 (M_r 11 700), and S-5 (M_r 9300) (Tamura et al., 1982). These subunits are assembled in such a manner as to be in conformity with the A–B structure proposed by Gill (1978). S-1 is the A protomer in a sense that it displays ADP-ribosyltransferase *A*ctivity when it is liberated from the holotoxin followed by reductive cleavage of the intrapeptide disulfide bridges (Katada et al., 1983). The residual subunits form a B oligomer, which is responsible for *B*inding of the toxin molecule in particular sites on the mammalian cell surface allowing the A protomer to enter the cell across the plasma membrane (Tamura et al., 1983). The subunit assembly in the B oligomer is such that two dimers, dimer-1 composed of S-2 and S-4 and the dimer-2 from S-3 and S-4, are connected to each other by means of S-5. S-5 is hence referred to as a *C*onnecting or C subunit (Tamura et al., 1982).

The specific substrate of the A protomer catalyzed ADP-ribosylation is the guanine nucleotide binding regulatory protein (N_i or G_i) that mediates receptor-coupled inhibition of adenylate cyclase in cell membranes (Katada & Ui, 1982a,b; Kurose et al., 1983; Murayama & Ui, 1983, 1984). The ADP-ribosylated N_i fails to communicate between receptors and the adenylate cyclase catalytic protein any longer (Ui, 1984). Consequently, not only receptor-mediated decreases in cellular cAMP (or inhibition of membrane adenylate cyclase) were mostly abolished but also receptor-mediated increases in cellular cAMP (or activation of membrane adenylate cyclase) were occasionally potentiated by prior exposure of a variety of cells to IAP (Katada & Ui, 1979, 1980, 1981a,b; Hazeki & Ui, 1981; Katada et al., 1982; Murayama & Ui, 1983; murayama et al., 1983; Kurose & Ui, 1983). N_i also plays an indispensable role in biosignaling arising from Ca^{2+}-mobilizing receptors in certain cell types. Ca^{2+}-related cellular responses to receptor agonists were abolished by the treatment of cells with IAP. These responses included compound 48/80-induced histamine secretion from mast cells (Nakamura & Ui, 1983–1985), formyl peptide induced superoxide anion generation in neutrophils (Okajima & Ui, 1984; Okajima et al., 1985; Ohta et al., 1985), thrombin-induced arachidonic acid release from 3T3 fibroblasts (Murayama & Ui, 1985), and opiate-induced cGMP production in NG108-15 hybrid cells (Kurose & Ui, 1985). Thus, multiple biological actions of pertussis toxin have been safely accounted for by the ADP-ribosyltransferase activity of the A protomer entering the cells by the aid of the B oligomer moiety of the toxin.

The B oligomer exhibited an additional important function apart from its function as a transporter of the A protomer. It acted as a potent mitogen in lymphocytes, probably as a result of cross-linking of membrane surface glycoproteins through its firm binding via the constituent two dimers (Tamura et al., 1983). Neither of the isolated two dimers could stimulate cells by themselves. The B oligomer must bind to the cell surface to permit the transmembrane entrance of the

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[1] Abbreviations: IAP, islet-activating protein (pertussis toxin); N_i, guanine nucleotide regulatory protein involved in inhibition of adenylate cyclase; $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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A protomer in a manner different from its binding leading to mitogenicity, since these two types of binding displayed distinctly differential susceptibility to chemical modification of the protein molecule. The B oligomer was not mitogenic, but the A protomer still entered the cell, when the free amino groups of the lysine residues were blocked by acetamidination in the B oligomer moiety of IAP (Nogimori et al., 1984a,b). Both lymphocytosis-promoting and histamine-sensitizing activities of IAP were markedly impaired by acetamidination, suggesting that the toxin-induced mitogenicity, rather than ADP-ribosylation, is somehow responsible for these well-known actions of pertussis toxin (Nogimori et al., 1984b), though the underlying mechanism remains to be clarified.

Modification of the biological activities of IAP caused by reductive methylation of lysine residues (Cabacungan et al., 1982) was qualtitatively the same as the modification by acetamidination. Reductive methylation was, however, better than acetamidination as a modification means in that much less denaturation of protein occurred during the modification procedure at neutral pH. The resultant methylated IAP was decomposed into the A protomer, C subunit, and two dimers under certain conditions. Cross reconstitution of hybrid toxins possessing an original hexameric structure was then achieved with the use of these methylated components together with nonmethylated components originating from the native IAP. Study of these hybrid toxins revealed the relative importance of two dimers, dimer 1 and dimer 2, in the development of mitogenic and relevant biological activities of IAP, as will be described in this paper.

Materials and Methods

Materials. IAP was purified from the 2-day culture supernatant of *Bordetella pertussis* (Tohama strain, phase I) according to the procedure previously reported (Yajima et al., 1978a,b). Sephacryl S-200, DEAE-Sepharose CL-6B, CM-Sepharose CL-6B, and Ficoll 400 were the products of Pharmacia Fine Chemicals. The reagents for radioimmunoassay of cAMP and insulin were obtained from Yamasa Shoyu Co. (Chiba, Japan) and Dainabot Radioisotope Laboratories (Tokyo), respectively. [α-^{32}P]NAD (28 Ci/mmol), [1-^{14}C]glucose (4 mCi/mmol), and [6-^{3}H]thymidine (27 Ci/mmol) were purchased from New England Nuclear and Amersham. Pyridine–borane was a product of Aldrich Chemical Co. Other reagents were of analytical grade from commercial sources.

Reductive Methylation of IAP. Reductive methylation of amino groups of IAP was achieved by the method of Feeney and his colleagues (Geoghegan et al., 1981; Cabacungan et al., 1982). To the solution of 2 mg/mL IAP in 0.1 M phosphate buffer/2 M urea (pH 7.0) were added pyridine–borane (in methanol) and formaldehyde to make final concentrations of 15 and 20 mM (or otherwise in Figure 1A), respectively, and the mixture was maintained under nitrogen gas at room temperature for 2 h (or otherwise in Figure 1B). The methylation reaction was terminated by the addition of 1 M glycine. Where indicated, the reaction mixture was applied to a column of Sephacryl S-200 that was eluted with 0.1 M phosphate buffer/2 M urea (pH 7.0) to obtain the methylated IAP as a single peak. The native IAP was also applied to the same column under the same conditions for comparison.

Resolution of Native and Methylated IAP into Their Constituent Components and Preparation of Hybrid Toxins by Cross Reconstitution of These Native and Methylated Components. The purified IAP was resolved into the A protomer, dimer 1, dimer 2, and C subunit by exposure of the toxin to 5 M urea at 4 °C for 4 days (Tamura et al., 1982). (See the first paragraph in the introduction for the subunit structure of IAP.) These toxin components were purified chromatographically with a CM-Sepharose column (Tamura et al., 1982).

Since the methylated A protomer of IAP tended to be further decomposed during 4-day exposure to 5 M urea, the resolution of the methylated IAP into methylated components was achieved by the following two-step method under more mild conditions. First, the methylated IAP (40 mg) was dialyzed against 4 M urea/0.02 M phosphate buffer (pH 8.5) for 6 h at 4 °C. The methylated A protomer liberated during the dialysis was adsorbed to a column (1.5 × 10 cm) of DEAE-Sepharose and then eluted as a single peak with 200-mL linear gradients of 0–0.5 M NaCl in 4 M urea/0.02 M phosphate buffer at the flow rate of 15 mL/h. The methylated B oligomer passed through the column. As the second step, it was gradually resolved into the methylated dimer 1, methylated dimer 2, and methylated C subunit in 5 M urea/0.05 M phosphate buffer (pH 6.3) during 24-h maintenance at 4 °C. These components (30 mg of protein) were purified and isolated from each other by means of gradient elution from a column (1.2 × 15 cm) of CM-Sepharose with 0–0.2 M NaCl (400 mL with the flow rate of 14.6 mL/h) in the presence of 5 M urea/0.1 M phosphate buffer (pH 7.2).

The following two-step procedure was also adopted for cross recombination of these methylated and nonmethylated components. Equimolar amounts of dimer 1 (or methylated dimer 1), dimer 2 (or methylated dimer 2), and C-subunit (or methylated C subunit) were mixed in 0.1 M phosphate buffer/2 M urea (pH 7.0) and maintained at 4 °C overnight to reconstitute the hybrid B oligomer moiety composed of the native and methylated components. To this solution was then added an equimolar amount of the A protomer (or the methylated A protomer), and the mixture (3 mg of protein) was again kept for 24 h at 4 °C under gentle stirring. The hybrid holotoxin thus formed was eluted as a single peak, sharply separated from unassociated components, from a Sephacryl S-200 column (1.5 × 95 cm). The yields (%) of various hybrid toxins are recorded in Table III.

Electrophoresis. The homogeneity of the native and methylated IAP or hybrid toxins was studied by electrophoresis of these proteins (30 μg each) for 2 h in 7.5% polyacrylamide gel rods at pH 4.3 with an electric current of 4 mA/rod according to Reisfeld et al. (1962). For the purpose of subunit analysis (Figure 2), the protein samples had been heated at 100 °C for 5 min in 1% $NaDodSO_4$ before being electrophoresed on 10–30% polyacrylamide slab gels by the method of Laemmli (1970) with slight modifications (Tamura et al., 1982). After 14-h electrophoresis at 120 V, the gels were stained with Coomassie Brilliant Blue R-250. In the case of identification of ADP-ribosylated proteins (Figure 5), radiolabeled C6 cell membranes were dissolved by being heated in 1% $NaDodSO_4$/5% 2-mercaptoethanol/10% glycerol/62.5 mM Tris-HCl/0.02% bromophenol blue (pH 6.8) for 3 min at 100 °C and electrophoresed on 12.5% polyacrylamide slab gels. The gels were stained, dried, and autoradiographed at −80 °C with Kodak X-Omat film for 24 h (Katada & Ui, 1982a,b).

Protein and Amino Acid Analysis. The protein concentration in the eluate from chromatographic columns was determined by the procedure of Lowry et al. (1951) with bovine serum albumin as standard. Free amino groups in the protein were determined by the method of Habeeb (1966) after complete dialysis of the protein sample (500 μg/mL) against

distilled water. The sample (0.4 mL) was mixed with equal volumes of 4% $NaHCO_3$ (pH 9.0) and 0.1% trinitrobenzenesulfonic acid and maintained at 37 °C for 1.5 h. The incubation was continued for an additional 10 min with the addition of 0.4 mL of 10% $NaDodSO_4$. The optical density at 335 nm was measured immediately after the final addition of 0.4 mL of 1 N HCl.

Amino acid analysis of protein was conducted in a Hitachi 835-50 amino acid analyzer after 24-h hydrolysis at 115 °C in 4 N methanesulfonic acid (Pierce Chemicals). The peak of methylated lysine was quantitated with a correction factor of 0.822 as described by Means and Feeney (1968).

Assay of Biological Activities of IAP, Methylated IAP, or Hybrid Toxins. The procedures employed were essentially the same as those recently reported by Nogimori et al. (1984a,b), as briefly outlined below. Epinephrine-hyperglycemia inhibitory activity was measured in vivo on the basis of the difference in 1-h hyperglycemia induced by subcutaneously injected epinephrine (200 μg/kg of body weight) between toxin-treated and nontreated rats. Toxins (IAP, methylated IAP, or hybrid toxins) were injected intravenously at a dose of 1.5 μg/rat (130–150 g of body weight) 1 day before the epinephrine challenge. The increase in the number of circulating white blood cells 1 day (Figure 1) or various days (Figure 8) after this dose of toxin was determined, without epinephrine challenge, as a measure of lymphocytosis-promoting activity. The increase was detected in lymphocyte fractions only.

Membranes were prepared from rat C6 cells that had been exposed to various concentrations of toxins for 3 h. GTP-dependent adenylate cyclase activities of these membrane preparations (30 μg of protein) were assayed in the reaction mixture (0.2 mL) of 50 mM Tris-HCl (pH 7.5)/5 mM $MgCl_2$/1 mM ATP/10 mM phosphocreating/20 units of creatine phospholinase/10 μM GTP/2 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)/0.4 mM dithiothreitol/1 mM 3-isobutyl-1-methylxanthine/0.4 mg of bovine serum albumin (Katada et al., 1982). Cyclic AMP generated during 5-min incubation at 37 °C was determined by a radioimmunoassay method (Honma et al., 1977). For ADP-ribosylation, membranes (200 μg of protein) from C6 cells not treated with toxin were incubated for 5 or 10 min with 50 mM Tris-HCl (pH 7.5)/5 mM $MgCl_2$/1 mM ATP/1 mM dithiothreitol/10 mM thymidine/10 μM [α-^{32}P]NAD (5 Ci/mmol) in the presence of various concentrations of toxins or their A protomers. The M_r 41 000 protein was separated and detected by $NaDodSO_4$–polyacrylamide gel electrophoresis and autoradiography as described above and counted for its ^{32}P content in a scintillation spectrometer (Katada & Ui, 1982a,b).

Adipocytes [(1–2) × 10^5 cells in 1 mL) prepared from rat epididymal fat tissues were incubated in Krebs–Ringer bicarbonate solution (pH 7.4) containing 2% bovine serum albumin under a gas mixture of 95% O_2/5% CO_2 for 3 h with toxins for the determination of glycerol release or first for 30 min without and then for 2 h with 0.2 mM [1-^{14}C]glucose (0.025 μCi) plus toxins (or insulin) for determination of glucose oxidation. For the latter determination, incubation was terminated by the addition of 20% perchloric acid, and $^{14}CO_2$ trapped by hyamine was counted. The toxin-induced reversal of adrenergic inhibition of insulin secretion from rat pancreas was also studied in vitro. Two pancreatic islets were incubated in 0.5 mL of TCM 199 culture medium fortified with fetal calf serum (10%), aprotinin (100 kallikrein inhibitory units), epinephrine (1 μg), and glucose (16.7 mM) in the presence of various concentrations of toxins for 6 h at 37 °C under 95% air/5% CO_2. Insulin released was assayed radioimmunochemically (Katada & Ui, 1979, 1981a,b).

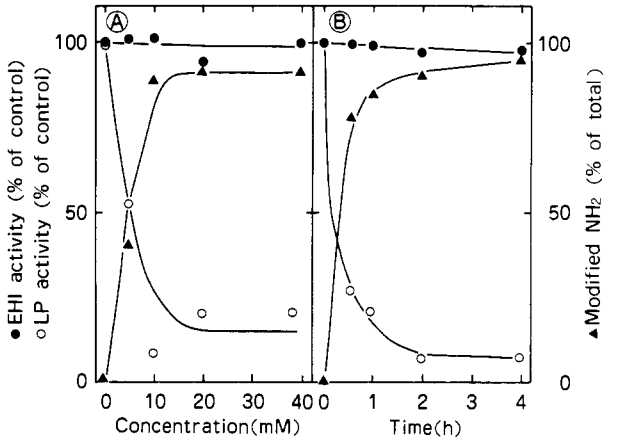

FIGURE 1: Reductive methylation of IAP. IAP was methylated as described under Materials and Methods with increasing concentrations of formaldehyde for 2 h (A) or with 20 mM formaldehyde for increasing times of incubation (B). The reaction mixture was then applied to a column of Sephacryl S-200 to obtain the modified protein, which was assayed for free amino groups and epinephrine-hyperglycemia inhibitory (EHI) and lymphocytosis-promoting (LP) activities as described under Materials and Methods. EHI and LP activities of modified IAP were expressed as percentages of the activities of the unmodified protein. The modified NH_2 represents the percent decrease in the free amino groups in the IAP molecule as induced by the methylation procedure. Each point is a mean from duplicate observations.

Mitogenic action of toxins and their B oligomers was evaluated with mouse splenic cells (2 × 10^6 cells in 1 mL), which were incubated in RPMI 1640 culture medium containing 5 mM 4-(2-hydroxyethyl)-2-piperazineethanesulfonate (Hepes, pH 7.4) and 10% fetal calf serum in the presence of various concentrations of toxins for 48 h at 37 °C under 95% air/5% CO_2. [^{3}H]Thymidine (0.8 μCi) was added at 24 h, and the radioactivity taken up by cells during the subsequent 24-h incubation was determined in a scintillation spectrometer. Hemagglutinin activity of methylated and nonmethylated IAP and their components was studied with 1% chicken erythrocytes in 0.9% NaCl/0.1% bovine serum albumin. Agglutination of blood cells induced by these proteins was checked by the naked eye after 1 h at 37 °C.

RESULTS

Preparation of Methylated IAP and Its Biological Activities. Incubation of IAP with reagents for reductive methylation, formaldehyde and pyridine–borane, at room temperature caused rapid disappearance of free amino groups in the protein in a manner dependent on time and formaldehyde concentrations (Figure 1). The decrease in free amino groups was actually due to dimethylation of lysine residues in protein as evidenced in Table I, which also showed that there was no significant difference in other amino acid composition between the native and modified IAP. The representative biological activities in vivo of IAP were also studied by injecting the thus modified toxin into rats. The degree of reductive methylation of amino groups was well correlated with an accompanying decrease in the lymphocytosis-promoting activity of the toxin without significant change in its epinephrine-hyperglycemia inhibitory activity (Figure 1). Just the same selective effects were provoked by acetamidination, too, on these two biological activities of the toxin (Nogimori et al., 1984a).

The reaction mixture from reductive methylation of IAP was submitted to gel filtration through Sephacryl S-200; the

Table I: Amino Acid Composition of Methylated IAP and Its Constituent Monomers and Dimers in Comparison with That of the Corresponding Unmodified Proteins

	residues (% of total) with (+) or without (−) methylation									
	IAP		A protomer		dimer 1		dimer 2		C subunit	
	−	+	−	+	−	+	−	+	−	+
Asp	7.1	6.9	9.4	9.3	6.1	5.5	6.0	5.5	8.5	8.8
Thr	7.1	7.0	7.0	6.8	7.5	6.6	6.6	6.2	6.1	6.3
Ser	6.3	6.1	7.9	6.7	6.5	5.9	5.3	4.8	5.7	5.9
Glu	8.8	8.9	10.3	9.7	8.3	8.4	8.5	8.6	8.8	9.0
Gly	8.9	9.0	8.7	8.4	9.9	8.9	9.4	9.0	8.2	8.3
Cys	1.6	1.7	1.2	1.8	2.0	2.1	2.1	2.0	2.2	2.1
Ala	9.9	10.0	11.3	10.2	7.3	8.0	10.3	10.4	10.1	10.1
Val	6.7	6.8	7.1	6.5	7.7	8.9	7.2	8.1	4.2	4.2
Met	2.5	2.7	1.6	1.8	2.7	3.9	2.3	3.1	1.7	1.2
Ile	3.9	4.0	3.4	3.3	3.9	3.3	4.2	4.0	3.6	2.9
Leu	8.1	8.2	5.1	6.0	8.5	9.0	8.7	9.2	13.4	14.9
Tyr	6.6	6.7	7.5	8.0	5.8	4.6	6.7	6.0	5.0	4.1
Phe	3.4	3.5	3.2	3.7	3.5	3.9	3.2	3.6	4.5	4.6
Lys	3.9	0.3	1.0	0.4	5.2	0.6	4.7	0.6	5.0	0.2
DM-Lys[a]		3.0		0.6		4.9		4.2		4.9
His	1.6	1.6	1.7	2.2	1.4	1.2	0.8	1.0	2.6	2.6
Arg	6.9	7.4	9.3	9.0	5.9	5.8	6.7	6.2	3.6	3.4
Trp	0.4	0.5	0.8	0.5	nd[b]	nd	nd	nd	0.8	0.6
Pro	6.0	6.1	3.6	5.3	7.4	8.3	7.0	7.6	5.5	5.8

[a] Dimethylated lysine. [b] nd, not determined.

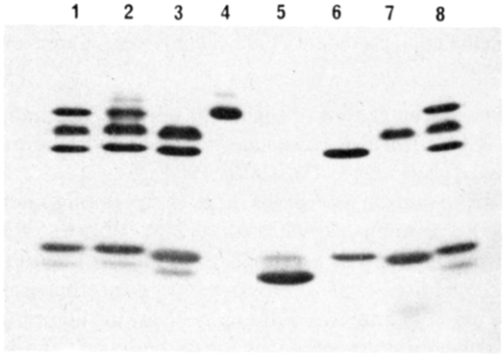

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of IAP, methylated IAP, and the monomers and dimers of methylated IAP. The native and methylated IAP and the components eluted from DEAE-Sepharose and CM-Sepharose columns (see Materials and Methods) were electrophoresed as described. (Lanes 1 and 8) Native IAP (16 μg); (lane 2) methylated IAP (16 μg); (lane 3) B oligomer (16 μg) that passed through the DEAE-Sepharose column; (lane 4) A protomer (4 μg) adsorbed by and eluted from DEAE-Sepharose; (lane 5) C subunit (10 μg) eluted from CM-Sepharose; (lane 6) dimer 2 (10 μg) eluted from CM-Sepharose; (lane 7) dimer 1 (10 μg) eluted from CM-Sepharose.

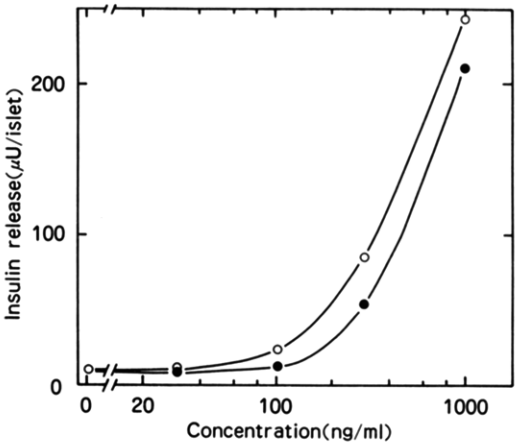

FIGURE 3: Reversal by the native or methylated IAP of epinephrine-induced inhibition of insulin secretion from rat pancreatic islets. Islets were incubated with epinephrine and glucose in the presence of the native (○) or methylated (●) IAP for 6 h to measure insulin release as described under Materials and Methods. Each point is the mean of duplicate observations.

methylated IAP was eluted as the main peak at the same position as the native IAP, although it was followed by a small peak of the mixture of methylated monomers and dimers, which were the decomposition products during dimethylation reaction (data not shown). The methylated IAP thus purified exhibited a sharp band with the same rate as the native IAP on polyacrylamide disc gel electrophoresis (not shown). Moreover, when the methylated IAP was disintegrated into subunits in $NaDodSO_4$, all the subunits, S-1 to S-5, migrated on a slab gel at just the same rates as did the corresponding subunits from the native IAP (Figure 2, lanes 1 and 2). Thus, physicochemical or electrochemical properties of IAP or its constituent subunits appeared not to be significantly affected by dimethylation of their lysine residues.

The IAP-induced inhibition of epinephrine hyperglycemia (as in Figure 1) was a reflection of hyperinsulinemia resulting from reversal of the catecholamine-induced inhibition of insulin secretion (Katada & Ui, 1976, 1977; Yajima et al., 1978b). In fact, the methylated IAP was almost as effective as, or only slightly less effective than, the native IAP in reversing epinephrine-induced inhibition of insulin release when the toxins were directly added to the incubation medium of rat pancreatic islets (Figure 3). Epinephrine inhibits adenylate cyclase via N_i in pancreatic islets (Katada & Ui, 1981a) as well as in other cells; the decrease in cellular cAMP is then responsible for inhibition of insulin secretion (Katada & Ui, 1979, 1980, 1981b; Yamazaki et al., 1982). These N_i-mediated cellular functions were abolished when N_i was ADP-ribosylated by the A protomer that had entered the cells. Thus, the sequence of events leading to the IAP-induced loss of N_i functions, i.e., the insertion of the toxin into the cells via binding of its B oligomer moiety followed by the liberation of the active A protomer (cellular processing) and the ADP-ribosylation of N_i by the processed A protomer, must occur with the methylated IAP as well. This was confirmed with the use of C6 cells in Figure 4.

The membrane M_r 41 000 protein, the α-subunit of N_i (Bokoch et al., 1984; Katada et al., 1984a,b), was ADP-ribosylated by the methylated IAP as well as the native IAP when C6 cell membranes were incubated with these toxins in the presence of radioactive NAD (Figure 4B). Likewise, the

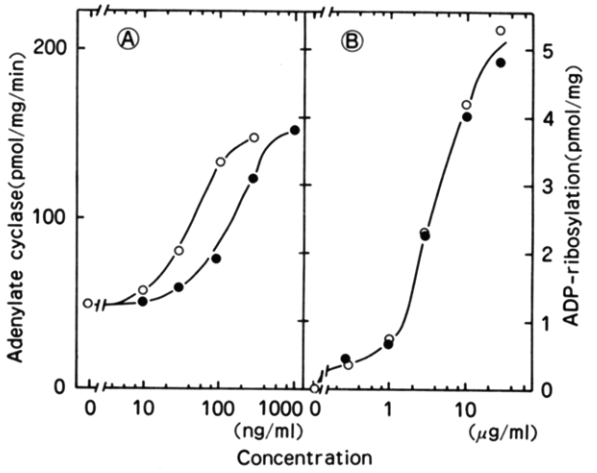

FIGURE 4: Abilities of the native and methylated IAP to increase GTP-dependent adenylate cyclase and to catalyze ADP-ribosylation of a membrane protein in rat glioma C6 cells. (A) Membranes prepared from the cells that had been exposed to the native (○) or methylated (●) IAP in increasing concentrations were assayed for adenylate cyclase activity in the presence of GTP. (B) Membranes prepared from C6 cells not exposed to the toxin were incubated with [α-^{32}P]NAD in the presence of the native (○) or methylated (●) IAP. The membrane M_r 41 000 protein, electrophoretically isolated as described under Materials and Methods, was then analyzed for its ^{32}P content. Each point represents the mean of duplicate observations.

GTP-dependent adenylate cyclase activity was higher in membranes from IAP-treated or methylated IAP-treated cells than in membranes from nontreated cells (Figure 4A). Slightly less effects of the methylated IAP than of the native IAP on intact cells (Figures 3 and 4A) in comparison with their equal effectiveness on membranes (Figure 4B) may suggest that the A protomer transporting activity of the B oligomer was slightly, but the ADP-ribosyltransferase activity of the A protomer was not at all, impaired by dimethylation of lysine residues in these components of IAP.

Resolution of Methylated IAP into Its Components. The methylated IAP was resolved into its components by the two-step exposure to urea as described under Materials and Methods. The methylated B oligomer purified by DEAE-Sepharose chromatography consisted of four subunits corresponding to methylated S-2 to S-5 on an NaDodSO$_4$–polyacrylamide gel electrophoretogram (Figure 2, lane 3), whereas the S-1 subunit was the only component of the methylated A protomer (Figure 2, lane 4). The methylated B oligomer was further disintegrated into methylated dimer 1 (lane 7), methylated dimer 2 (lane 6), and methylated C subunit (lane 5), all of which showed the same migration rates as the corresponding nonmethylated subunits upon electrophoresis (Figure 2, lanes 1 and 8). The analaysis of the amino acid composition showed that 60 (A protomer) to 98% (C subunit) of the lysine residues of these components of IAP were dimethylated (Table I). The degree of dimethylation was roughly the same as the degree of acetamidination previously reported (Nogimori et al., 1984a).

Biological Activities of Methylated A Protomer, Methylated B Oligomer, and Methylated Dimers. The methylated A protomer and the methylated B oligomer were studied for their biological activities. The transfer of the ADP-ribose moiety of NAD to the C6 cell membrane M_r 41 000 protein as catalyzed by the A protomer was not affected by methylation of its lysine residues (Figure 5). As much the degree of ADP-ribosylation as that caused by the nonmethylated A protomer was caused by the methylated A protomer, regardless of whether it was derived from the methylated IAP (lane 4) or the A protomer from the native IAP methylated under the same conditions as employed in Figure 1 (lane 3). Moreover, methylated IAP itself was as effective as the methylated or nonmethylated A protomer of IAP in this regard (lane 5). This finding confirmed the above-mentioned conclusion that not only the ADP-ribosyltransferase activity of the A protomer but also the susceptibility of the holotoxin to processing to liberate the active A protomer during incubation with C6 cells survived the procedure of dimethylation of the IAP molecule.

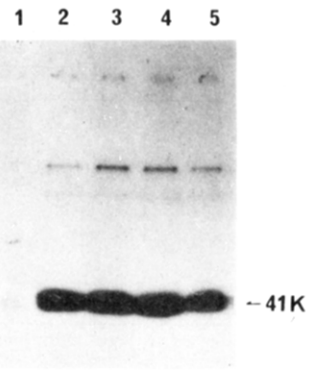

FIGURE 5: ADP-ribosylation of cell membrane proteins by the A protomer of the native and methylated IAP. Membranes from C6 cells were incubated with [α-^{32}P]NAD and the following additions for 10 min at 37 °C. The labeled membranes were then dissolved in NaDodSO$_4$, electrophoresed, and autoradiographed as described under Materials and Methods. The additions were none (lane 1), the A protomer from the native IAP (lane 2), the A protomer isolated from the native IAP and methylated under the same conditions as the methylation of IAP (lane 3), the A protomer from methylated IAP (lane 4), and methylated IAP (lane 5). The concentrations of additions were 25 μg/mL for lanes 2–4 and 50 μg/mL for lane 5. An arrow shows the band of protein with an M_r of 41 000.

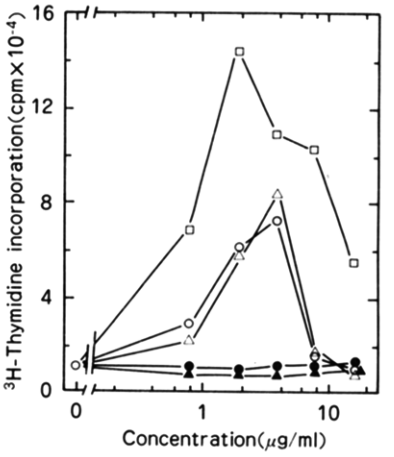

FIGURE 6: Mitogenic actions of methylated IAP and its B oligomer. Mouse splenic cells were incubated with various concentrations of IAP (○) or its B oligomer (△), methylated IAP (●) or its B oligomer (▲), or concanavalin A (□) for 48 h. [^{3}H]Thymidine was added to the cell suspension at 24 h to determine the subsequent incorporation of radioactivity into cells as an index of mitogenicity as described under Materials and Methods. Each point (incorporation per 10^6 cells) represents the mean of duplicate determinations.

The isolated B oligomer of IAP was as effective as IAP itself in mitogenicity observable with mouse splenic T cells (Tamura et al., 1983). These mitogenic activities were abolished completely by reductive methylation (Figure 6), confirming our previous proposal, based on the similar effect of acetamidination (Nogimori et al., 1984b), that different mechanisms are involved in the mitogenic and the A protomer transporting activities of the B oligomer. The free amino groups in lysine residues play an indispensable role in the mitogenic action, but not in the transporting action, of the B oligomer.

Table II: Hemagglutinin Activity of Native and Methylated IAP and Their Components[a]

additions	methylated	250	125	62.5	31.3	15.6
none (vehicle)		−	−	−	−	−
IAP	no	+	+	+	±	−
	yes	+	+	+	±	−
B oligomer	no	+	+	+	±	−
	yes	+	+	+	±	−
dimer 1	no	+	+	+	±	−
	yes	+	+	±	−	−
dimer 2	no	±	−	−	−	−
	yes	−	−	−	−	−

[a] (+) Blood cells agglutinated; (−) no agglutination observed; (±) marginal agglutination.

As exemplified in Figure 6, some of the diverse biological activities in vitro of IAP were reproduced by the B oligomer. Neither of the two dimers constituting the B oligomer, however, was usually effective on cells by itself. The only exception was the toxin-induced hemagglutination (Table II). The hemagglutinin activity of IAP was mimicked not only by the B oligomer but also by dimer 1, whereas dimer 2 was barely effective in this regard (Table II). Essentially, the same effects were observed with methylated proteins, indicating that the free amino groups of lysine residues were not essential for the hemagglutination to be induced by the toxin or the toxin components.

Preparation of Hybrid Toxins Composed of Nonmethylated and Methylated Components. The complete subunit assembly of the IAP holotoxin was readily reconstituted by maintaining the mixture of the purified components, the A protomer, dimer 1, dimer 2, and C subunit, in 2 M urea at 4 °C (Tamura et al., 1982). The same hexameric structure was also obtained by the same simple procedure even if some of these four components were replaced by methylated ones. The four kinds of hybrid toxins thus obtained are listed in Table III; they are henceforth referred to as H-AC, H-D1, H-D2, and H-DD to symbolize the methylated components (A, A protomer; C, C subunit; D, dimer) following H (hybrid). These hybrid toxins migrated as sharp bands at the same rate on polyacrylamide disc gel electrophoresis and were separated into five subunit bands upon $NaDodSO_4$–polyacrylamide gel electrophoresis (not shown). Their stability in 2 M urea was not significantly different from that of the native IAP.

Biological Activities of Hybrid Toxins—Differential Roles of Two Dimers in Cell Stimulation. The rate of glycerol release in vitro from adipocytes is very low because adenosine endogenously generated during dispersion of the cells inhibits, via N_i, adenylate cyclase producing cAMP, which is a mediator of glycerol liberation from triglycerides. The suppression of the N_i function by exposure of adipocytes to IAP, therefore, resulted in enhanced glycerol release from the cells (Tamura et al., 1983; Nogimori et al., 1984b). Thus, stimulation of glycerol release from adipocytes, like insulin release from islets, is one of the assay systems for ADP-ribosylation of N_i by the A protomer transported and processed after the binding of IAP via the B oligomer to the cells. All the hybrid toxins tested as well as the methylated IAP caused the same degree of maximal glycerol release as did the native IAP in a manner similarly dependent on their concentrations from 1 to 200 ng/mL (data not shown). Thus, the previous conclusion was again confirmed that the free amino groups in peptides are not essentially required for the A protomer transporting activity of the B oligomer of IAP.

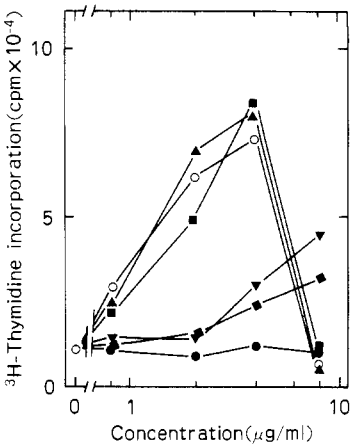

FIGURE 7: Mitogenic actions of the native IAP, methylated IAP, and hybrid toxins. Mouse splenic cells were incubated for 48 h with various concentrations of the native IAP (○), methylated IAP (●), H-AC (▲), H-D1 (■), H-D2 (▼) or H-DD (♦), and [^{3}H]thymidine was added at 24 h of incubation to study the incorporation of ^{3}H into the cells as described under Materials and Methods. Each point (incorporation per 10^6 cells) is the mean of duplicate observations.

The free amino groups of the two dimers played, however, different roles in mitogenic action of IAP (Figure 7). H-AC and H-D1 were mitogens as potent and efficacious as the native IAP, whereas H-D2 and H-DD did not stimulate DNA synthesis significantly even at 4 μg/mL, the concentration eliciting the maximal synthesis in the case of the native IAP. Thus, the methylation of dimer 2 did, but the methylation of dimer 1 or C subunit did not, interfere with the mitogenicity of the B oligomer. It was suggested that the free amino groups in dimer 2 may play an important role in binding of the B oligomer to stimulate lymphocytes.

Just the same influences were exerted by hybrid toxins upon the number of circulating leukocytes in vivo (Figure 8). H-AC and H-D1 were as effective as the native IAP; the lymphocytosis-promoting activity of the toxin was not impaired by methylation of subunits other than dimer 2. In sharp contrast, H-D2 and H-DD were much less effective than the native IAP; the free amino groups in dimer 2 must be required for the lymphocytosis-promoting activity of IAP in vivo. It is very likely that the direct mitogenic action of IAP on lymphocytes is somehow responsible for developments of lymphocytosis in vivo after the injection of the toxin into animals.

Differential Antagonism of Actions of IAP on Adipocytes and Lymphocytes by Dimers and Methylated Dimers. The foregoing results with methylated IAP and hybrid toxins have

Table III: Compositions, Yields, and Mitogenic Activities of Hybrid Toxins

abbreviations[a]	methylation of components				yield (%)	mitogenic act.[b]
	A protomer	dimer 1	dimer 2	C subunit		
H-AC	yes	no	no	yes	45.8	+
H-D1	no	yes	no	no	42.9	+
H-D2	no	no	yes	no	57.6	−
H-DD	no	yes	yes	no	30.1	−

[a] Abbreviations of hybrid toxins are such that H (hybrid) is followed by the components that are methylated. A, C, D1, D2, and DD represent the A protomer, C subunit (S-5), dimer 1, dimer 2, and both dimers, respectively. [b] The mitogenic activity of hybrid toxins at 2–4 μg/mL shown in Figure 7 is listed: (+) active; (−) inactive.

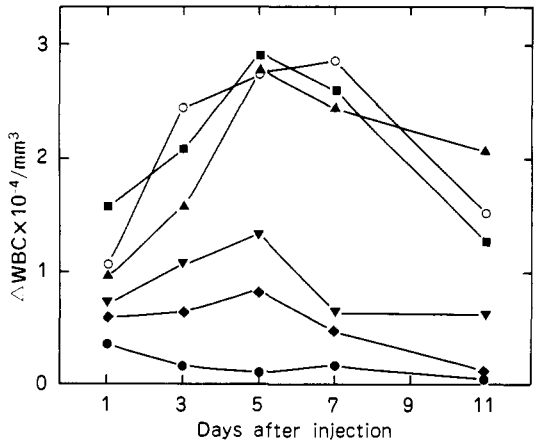

FIGURE 8: Lymphocytosis-promoting actions of the native IAP, methylated IAP, and hybrid toxins. Rats were injected intravenously with 4 μg of the native IAP (○), methylated IAP (●), H-AC (▲), H-D1 (■), H-D2 (▼), or H-DD (♦), and the increases in circulating leukocytes (WBC) were measured after various days elapsed. See Materials and Methods for details of experimental procedures. Each point (an increase in the cell number per microliter of whole blood) is the mean from four animals.

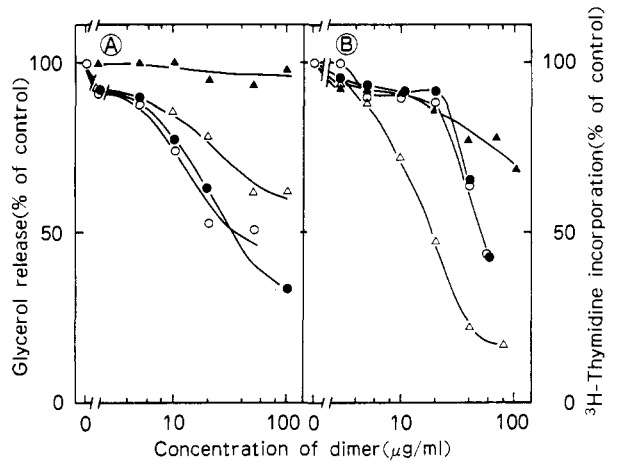

FIGURE 9: Inhibition of the IAP actions to stimulate glycerol release and thymidine incorporation by dimers or methylated dimers of IAP. Rat adipocytes (panel A) or mouse splenic cells (panel B) were incubated with various concentrations of dimer 1 (○), dimer 2 (△), methylated dimer 1 (●), or methylated dimer 2 (▲) for 10 min. These cells were further incubated for 3 h with 30 ng/mL of IAP to measure glycerol release (A) or for 48 h with 4 μg/mL of IAP to measure the incorporation of thymidine (added at 24 h after the addition of IAP) into cells (B). Each point, the mean of duplicate observations, is a percentage of the control value, which was obtained with IAP alone. See Materials and Methods for details of experimental procedures.

suggested that two dimers, dimer 1 and dimer 2, play apparently different roles in insertion of the A protomer into cells, e.g., stimulation of glycerol release from adipocytes, on the one hand and in strong binding of the B oligomer, e.g., mitogenicity in lymphocytes, on the other hand. Binding of these dimers to the cell surface was then studied more directly on the basis of the dimer-induced inhibition of these two IAP actions on intact cells. IAP-induced glycerol release from adipocytes was suppressed progressively as the concentration of dimer 1 or dimer 2 was increased (Figure 9A). Dimer 1 was more potent than dimer 2. The difference in potency between two dimers was more striking after they were methylated; the methylated dimer-1 was as potent as the non-methylated dimer-1 while the methylated dimer-2 was without effect at all up to the highest concentration (100 μg/mL) employed (Figure 9A).

In sharp contrast, dimer 2 was 4 times more potent than dimer I in antagonizing the mitogenic action of IAP in lymphocytes (Figure 9B). The relation of dimer 2 to dimer 1 in potency, however, was reversed by methylation; methylated dimer 2 was less effective than methylated dimer 1 at concentrations higher than 20 μg/mL, since the dimer 2 induced antagonism was markedly reduced, but dimer 1 induced one was not reduced at all, by methylation (Figure 9B). Thus, the free amino groups in protein molecules are required for the binding of dimer 2 to the cell surface. This binding via dimer 2 is likely to play more significant roles in stimulation of cells such as lymphocytes than in insertion of the A protomer into cells such as adipocytes.

Discussion

Reductive methylation of free amino groups in the IAP molecule caused profound modification of some of its biological activities. Most of the ε-amino groups of lysine residues in the protein were actually dimethylated under the experimental conditions employed (Table I). The α-amino groups at N-termini are known to be more readily methylated by formaldehyde in the presence of a reducing agent at neutral pH. Haas and Rosenberg (1985) have recently reported that most of the α-amino groups, but only 10% (or less) of the ε-amino groups, were modified when various proteins were incubated with 0.2 M formaldehyde at pH 7.0 for 15 min at 37 °C. In our experiments, incubation of IAP with 5 M formaldehyde for 2 h caused modification of about half of the total free amino groups and about half-maximal inhibition of lymphocytosis-promoting activity of the toxin (Figure 1A). More inhibition was observable when the concentration of formaldehyde was further increased. Essentially all of the reactive N-terminal amino groups must have been modified under these conditions. Thus, dimethylation of ε-amino groups of lysine residues, rather than that of α-amino groups, is likely to be responsible for altered biological activities of the modified IAP described in this paper.

The binding of the B oligomer moiety of IAP to the cell surface triggers dual actions as briefly described in the introduction. First, the binding allows the A protomer to enter cells, leading to APD-ribosylation of N_i located on the intracellular side of the plasma membrane. This action is responsible for increases in GTP-dependent adenylate cyclase activity in membranes, reversal of α_2-adrenergic inhibition of insulin secretion in pancreatic islets, or glycerol release from adipocytes. Second, the B oligomer causes mitosis of T lymphocytes (Tamura et al., 1983). The mitogenic action of IAP or its B oligomer was totally abolished by methylation of free amino groups in the IAP or B oligomer molecule (Figure 6). In sharp contrast, membrane adenylate cyclase activity (Figure 4A), insulin secretory responses of islets (Figure 3), or glycerol releases from adipocytes were enhanced by the methylated IAP as well as the native IAP. Thus, the present results have confirmed our previous conclusion that distinctly different mechanisms are involved in the above-mentioned two types of binding of the B oligomer to cells, showing that the free amino groups in the B oligomer moiety of IAP are indispensable for the binding leading to mitogenicity but not essential for the binding prior to the transmembrane insertion of the A protomer.

Reductive methylation was superior to acetamidination as the means of chemical modification of IAP molecules in that the modification proceeded under more mild conditions. As a result, methylated IAP was very similar to the unmodified IAP in their physicochemical properties. It consisted of methylated subunits that could be isolated or reconstituted to

the original hexameric structure under the similar conditions employed for isolation or reconstitution of the unmodified subunits. Taking advantage of such stability of methylated subunits, four kinds of hybrid IAP molecules were prepared from methylated and nonmethylated components (Table III), to further study the differential roles of free amino groups in these components in development of the biological activities of IAP. The results obtained with these hybrid toxins will be simply summarized below.

The hybrid toxins in which dimer 2 was not methylated (H-AC and H-D1) were as effective as the native IAP, while the toxins possessing methylated dimer 2 (H-D2 and H-DD) were essentially without effect at moderate concentrations, in eliciting mitogenicity in lymphocytes (Figure 7, also simply recorded in the last column of Table III). It is very likely, therefore, that the free amino groups in dimer 2 do, but the amino groups in dimer 1 do not, play important roles in firm binding of the B oligomer to cause mitosis. This conclusion was supported by the competition of dimers with IAP for its mitogenic action in Figure 9B; the dimer 1 induced weak antagonism was not virtually affected, but dimer 2 induced more potent antagonism was strongly attenuated, by reductive methylation of the respective dimers.

Either dimer 1 or dimer 2 was also antagonistic, though only partially, to the action of IAP to stimulate glycerol release from adipocytes. This reflects the binding of the B oligomer via these dimers as the first step of entrance of the A protomer moiety into adipocytes. In this case, however, dimer 1 was conversely more potent than dimer 2, indicating that the binding via dimer 1 was more important than the binding via dimer 2 in transmembrane insertion of the A protomer into the cells. Methylated dimer 2 did not antagonize the lipolytic action of IAP despite the fact that methylated dimer 1 was as effective as nonmethylated dimer 1 as an antagonist of this IAP action. The reductive methylation was also without effect on the hemagglutinin activity of IAP or its B oligomer, which appeared to reflect their binding to erythrocytes principally via dimer 1 (Table II). Thus, the free amino groups readily susceptible to reductive methylation appear to be located in the peptide domain that is responsible for binding in dimer 2 and in the domain not responsible in dimer 1.

The above-mentioned failure of methylated dimer 2 to antagonize the lipolytic action of IAP on adipocytes (Figure 9A) may suggest that this dimer is not capable of binding to the cell surface after being methylated. Nevertheless, H-D2 and H-DD, the hybrid toxins in which dimer 2 was methylated, were essentially as effective as the native IAP in stimulating glycerol release from adipocytes. It is likely, therefore, that dimer 1 and dimer 2 in the B oligomer moiety of IAP occupy the same sites on adipocytes or other cells to introduce the A protomer moiety into the cells. In other words, the "monovalent" binding of the B oligomer via either dimer 1 or dimer 2 to the same sites must be sufficient for the entrance of the associated A protomer into the cells. The affinity for these sites was higher with dimer 1 than with dimer 2 (Figure 9A). Hence, lipolytic action of IAP would be antagonized by either dimer 1 or dimer 2 as observed in Figure 9A, while hybrid toxins in which dimer 1 and/or dimer 2 was methylated were lipolytic agents as potent as native IAP, because methylated dimer 1 was able to bind to the sites on the cells with the same potency as the nonmethylated dimer 1 (Figure 9A).

On the other hand, H-DD was, like H-D2, a very weak mitogen (Figure 7) despite the lack of effects of methylation on the capability of dimer 1 to bind to lymphocytes (Figure 9B). Thus, "divalent" binding via both dimer 1 and dimer 2 to lymphocytes was essential for stimulation of the cells to cause mitosis, though the binding affinity was higher with dimer 2 than with dimer 1 in this case (Figure 9B). The concentration of IAP needed for mitogenicity in lymphocytes was 100–1000-fold higher than its concentration to cause the ADP-ribosylation of N_i. Probably, cross-linking of membrane glycoproteins resulting from divalent binding of large amounts of the toxin is responsible for mitogenicity to be induced. The insulin-like action of IAP to stimulate glucose oxidation in adipocytes could be also explained by the divalent binding rather than the monovalent binding, since H-D2 was much less effective than native IAP in this regard (data not shown).

The lymphocytosis-promoting activity is well-known to be unique to pertussis toxin, though little is known as yet of the mechanism involved. We have previously proposed that mitogenic activity of the toxin is responsible for its lymphocytosis-promoting activity, on the basis of the finding that both activities were abolished by acetamidination of the toxin molecule (Nogimori et al., 1984a,b). It is further shown in this paper that both actions of IAP were modified by methylation of constituent dimers in a strictly parallel fashion (compare Figure 7 with Figure 8). The hybrid toxins with methylated dimer 2, like methylated IAP, were not mitogenic and produced barely detectable lymphocytosis, while the hybrid toxins with nonmethylated dimer 2 were potent mitogens and increased the number of circulating lymphocytes enormously when injected into rats. Thus, further evidence has been provided with hybrid toxins for an involvement of mitogenicity in lymphocytosis promotion induced by IAP.

In summary, the B oligomer moiety of IAP bound to the mammalian cell surface in dual manners. The monovalent binding via either dimer 1 or dimer 2 was the essential step for the subsequent entry of the A protomer moiety into numerous types of cells, whereas the divalent binding via both dimers occurred necessarily in lymphocytes. The divalent binding of rather large amounts of the toxin resulted in mitosis of lymphocytes, which must be causally related to the toxin's well-known lymphocytosis-promoting activity in vivo. The free amino groups in the lysine residues play an essential role in binding of dimer 2 but not in binding of dimer 1. Thus, the chemically modified pertussis toxin in which the free amino groups are blocked will by promising as a selectively functional agent, e.g., a potentiator of insulin secretory responses without inducing advert lymphocytosis.

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Registry No. Insulin, 9004-10-8; adenylate cyclase, 9012-42-4; glycerol, 56-81-5.

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