Identification of the Predominant Substrate for ADP-Ribosylation by Islet Activating Protein*

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Islet activating protein (IAP), a toxin isolated from Bordetella pertussis, blocks the ability of inhibitory hormones to attenuate adenylate cyclase activity and enhances the ability of stimulatory hormones to activate the enzyme. The toxin appears to act by catalyzing the transfer of ADP ribose from NAD to a 41,000-dalton protein in target cell membranes. A protein purified from rabbit liver membranes, apparently composed of 41,000- and 35,000-dalton subunits, is shown to be a specific substrate for IAP. Cholera toxin does not ADP-ribosylate this protein. In contrast, the purified guanine nucleotide-binding regulatory component of adenylate cyclase (G/F), which is ADP-ribosylated by cholera toxin, is not covalently modified by IAP.

Equilibrium binding studies and photoaffinity labeling experiments demonstrate that the 41,000-dalton subunit of the IAP substrate has a specific binding site for guanine nucleotides.

IAP,† a toxin isolated from the culture medium of Bordetella pertussis (1-4), has been shown to exert effects on the adenylate cyclase system in vivo (5) and in a variety of intact cell preparations; the latter include cultured rat pancreatic islet cells (6), isolated rat heart cells (7), and rat C6 glioma cells (8). These effects include potentiation of receptor-mediated stimulation of cyclic AMP accumulation, as well as attenuation of receptor-mediated inhibition of cyclic AMP synthesis. Membranes prepared from IAP-treated pancreatic islet cells show a marked decrease in GTP-dependent AMP-activated adenylate cyclase (9). More recently, it has been demonstrated that direct addition of IAP to a membrane preparation from C6 glioma cells causes enhanced GTP-dependent adenylate cyclase activity (8, 10). The enhancement of GTP-dependent adenylate cyclase activity by IAP is correlated with the covalent incorporation of 32P from the ADP moiety of NAD into a membrane protein with an apparent molecular weight of 41,000 (11). This 41,000-dalton substrate for IAP differs in electrophoretic mobility from the 45,000-dalton substrate that is ADP-ribosylated by cholera toxin (11). ADP-ribosylation of the 41,000-dalton protein by IAP in NG108 neuroblastoma-gloma cell membranes also correlates with the loss of the ability of inhibitory hormones to attenuate adenylate cyclase activity.²

The purification of a guanine nucleotide-binding regulatory component of adenylate cyclase, termed G/F, has been described by this laboratory (12-14). We now report the identification and partial purification of an apparently related protein that contains two polypeptides with molecular weights of approximately 41,000 and 35,000. This protein can be specifically labeled by IAP in the presence of [32P]NAD and, in addition, is able to bind guanine nucleotides.

EXPERIMENTAL PROCEDURES

Cholera toxin was purchased from Calbiochem and was activated at 37 °C for 20 min in 25 mM potassium phosphate (pH 8.0) and 20 mM diethiothreitol immediately prior to use. Pure IAP and its S1 subunit were provided by Dr. Michio Ui and the Research Laboratories, Kakenyaku Kako Co., Shiga, Japan. L-a-dimyristoyl phosphatidylcholine was purchased from Sigma and was sonicated in 20 mM sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 8.0), 1 mM EDTA, and 2 mM MgCl₂ for 15-20 min prior to use. ATP, GTP, and NAD were also obtained from Sigma. [α-32P]ATP was synthesized according to the procedure of Johnson and Walseth (15). This radiolabeled nucleotide was utilized for the synthesis of [32P]NAD by the method of Casel and Pfeffer (16). E-Aspartyl-[32P]GTP was obtained from Schwarz-Mann, while [32P]GTP-S was purchased from New England Nuclear. ARF was prepared as described (17). Protein was assayed by an amido black staining procedure (18).

Purification of the IAP Substrate and of G/F-G/F was purified from rabbit liver and was assayed by procedures that have been described previously (13). When the heptazine-Sepharose chromatographic step for the purification of G/F is performed in the absence of ATP, Mg²⁺, and F⁻ (see Ref. 13), a protein fraction that consists predominantly of 41,000- and 35,000-dalton polypeptides is resolved from G/F. The properties of this fraction are described below, and details of the further purification of this protein will be provided in a subsequent publication.

Labeling with [32P]NAD—Radiolabeling of membranes with [32P]NAD and IAP (or its S1 subunit) was monitored by autoradiography. Membranes were incubated with [32P]NAD (10 μM, 5,000-15,000 cpm/μmole) in the presence of 100 mM Tris-HCl (pH 8.0), 10 mM thymidine, 1 mM ATP, 100 μM GTP, 2.5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, and the indicated concentration of IAP or its S1 subunit for 30 min at 30 °C, unless otherwise specified. At the end of the labeling period, samples were diluted 5-fold into Laemmli sample buffer (19). A similar protocol was utilized for incubation with activated cholera toxin, with the exception that 100 mM potassium phosphate buffer (pH 7.5) was substituted for Tris-HCl.

Radiolabeling of detergent-solubilized proteins was carried out essentially as described (17). Reactions that contained cholera toxin and ARF also included 100 mM potassium phosphate (pH 7.5), 10 mM thymidine, 100 μM GTP, 1 mM ATP, 2.5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 3 mM L-a-dimyristoyl phosphatidylcholine, and 10 μM NAD; reaction mixtures that contained IAP (or its S1 subunit) included 100 mM Tris-HCl (pH 8.0) in place of potassium phosphate. The addition of detergent-solubilized G/F or IAP substrate yielded final concentrations of cholera toxin of 0.1% or less. Incubations were generally for 1 h at 30 °C, and reactions intended for autoradiographic analysis were stopped by 5-fold dilution into Laemmli sample buffer. SDS-polyacrylamide gel electrophoresis and autoradiography were performed as described (17).

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carried out as described previously (17). For quantitative estimates of the incorporation of \(^{32}P\) into protein, samples were precipitated by the addition of 1% sodium dodecyl sulfate and 15% trichloroacetic acid and filtered under vacuum through B85 nitrocellulose filters (S and S). After washing with 6% trichloroacetic acid (10 x 2 ml), filters were then counted in a toluene-based scintillation cocktail.

Binding of Guanine Nucleotides—Binding of \(^{[35]S}\)GTPyS and 8-azido-[\(^{32}P\)]GTP were measured by described procedures (20).

Determination of Hydrodynamic Parameters—Hydrodynamic properties of the IAP substrate were examined with or without prior exposure of the protein to Mg\(^{2+}\) and GTPyS or F', ATP, and Mg\(^{2+}\). For gel filtration of untreated samples, Ultrogel AcA-44 columns (0.9 x 25 cm) were equilibrated and eluted with TED buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol), 0.8% sodium cholate, and 100 mM NaCl. After the IAP substrate had been incubated for 120 min at 30°C in TED buffer containing 0.1% Lubrol, 30 mM MgCl\(_2\), and 10 \(\mu\)M [\(^{32}S\)]GTPyS, the column buffer utilized for gel filtration also contained 30 mM MgCl\(_2\). When the IAP substrate had been incubated for 10 min at 30°C in TED buffer containing 0.1% Lubrol, 30 mM MgCl\(_2\), 1 mM ATP, and 10 mM NaF, the column buffer also contained these concentrations of MgCl\(_2\), ATP, and NaF. In each case, samples of the IAP substrate (1-2 \(\mu\)g) and calibrating markers were diluted in the appropriate buffer (400 \(\mu\)l total volume) and were applied to the columns. The flow rate was adjusted to 4-6 ml/h and 400-600 vol were collected.

Linear sucrose gradients (4 ml) were prepared from 5 and 20% (w/v) sucrose solutions in the same sets of buffers and ligands that were utilized for gel filtration. Samples containing the IAP substrate (1-2 \(\mu\)g) and calibrating markers were diluted into the appropriate solutions (400 \(\mu\)l total volume), applied to the gradients, and centrifuged at 4°C in a Beckman SW 60 Ti rotor at 60,000 rpm for 15 h. After centrifugation, 20-24 fractions of 9 drops each were collected.

The position of the untreated or the fluoride-treated IAP substrate in column eluates and sucrose gradients was detected by labeling with \(^{[32]P}\)NAD and IAP. Samples were then precipitated with trichloroacetic acid and filtered (see above); radioautography was performed in some experiments to identify the labeled product. Samples that had been incubated with \(^{[35]S}\)GTPyS were filtered for detection of bound nucleotide (20). Binding of GTPyS inhibits ADP ribosylation by IAP.) The preparation used for hydrodynamic studies contained approximately 80% of the protein as 41,000- and 35,000-dalton polypeptides; this preparation bound \(^{[35]S}\)GTPyS to the extent of 0.7 mol/75,000 g of protein and could be ADP-ribosylated to the same extent.

RESULTS

As has been described by Katada and Uii (10, 11), IAP catalyzes the incorporation of \(^{32}P\) from \(^{[32]P}\)NAD primarily into a 41,000-dalton protein in membranes prepared from rat C6 glioma cells (Fig. 1, lane 2). Labeling was dependent on the presence of ATP in the incubation medium (Fig. 1, lane 3), but was unaffected by the absence of GTP (Fig. 1, lane 4). Cholera toxin stimulated the labeling of the 52,000- and 45,000-dalton subunits of G/F in C6 membranes (Fig. 1, lane 5); these proteins were clearly different from the predominant substrate for IAP. Liver membranes also contained a 41,000-dalton substrate for IAP, as demonstrated by labeling with \(^{[32]P}\)NAD (Fig. 1, lane 6). In contrast, labeling of the two subunits of G/F by cholera toxin, a reaction that is known to occur with hepatic G/F (12), was difficult to demonstrate (Fig. 1, lane 7). This can be attributed to the high rate of hydrolysis of NAD by these membranes. IAP has been shown to have a much lower \(K_m\) for NAD than does cholera toxin (11).

When rabbit liver membranes are solubilized with sodium cholate and extracts are fractionated as described for the purification of G/F (13), there is coincidental or nearly coincidental chromatographic overlap of G/F IAP substrate activity during the first two steps (DEAE and AcA 34). However, during fractionation of this material on heptamylene-Sepharose (with the absence of ATP, Mg\(^{2+}\), and F'), the two activities are largely resolved (Fig. 2). Several fractions containing IAP substrate activity are essentially free of G/F. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of such fractions indicates that two polypeptides with molecular weights of 41,000 and 35,000 comprise the majority of the protein in this peak (Fig. 3, lane 8). Also shown in Fig. 3 (lane 9) are the polypeptides that constitute purified rabbit liver G/F, with molecular weights of 35,000, 45,000, and 52,000.

Labeling experiments were undertaken with the preparations of IAP substrate and of G/F shown in Fig. 3. The 41,000-dalton protein could be labeled in the presence of \(^{[32]P}\)NAD plus IAP (or its S subunit) (lane 7). Labeling did not occur

\(^3\)Rabbit liver G/F is believed to be a mixture of 45,000/35,000 and 52,000/35,000 heterodimers (13); the 52,000-dalton polypeptide is absent in turkey and human erythrocyte G/F (14).
in the absence of the toxin (lane 2). ARF, which has been shown to be a requirement for the cholera toxin-dependent ADP-ribosylation of purified G/F (17), did not markedly affect labeling of the 41,000-dalton polypeptide by IAP (lanes 6 and 7). The 41,000-dalton protein was not labeled by cholera toxin, either in the presence or absence of ARF (Fig. 3, lanes 3 and 4). Purified rabbit liver G/F (Fig. 3, lane 9) was not labeled by IAP in the presence or absence of ARF (Fig. 3, lanes 13 and 14). In contrast, cholera toxin catalyzed the ADP-ribosylation of the 52,000- and 45,000-dalton subunits of G/F in an ARF-dependent manner (Fig. 3, lanes 10–12).

The specificity of the labeling by IAP is further demonstrated in Fig. 4. In this experiment, a fraction (from the heptylamine-Sepharose column) was chosen that contained roughly equal amounts of G/F and the IAP substrate. In the presence of \[^{32}P\]NAD and IAP, only the 41,000-dalton protein was labeled; there was no labeling of the 45,000-dalton subunit of G/F either in the presence or absence of ARF (Fig. 4, lanes 4 and 5). Likewise, when cholera toxin and \[^{32}P\]NAD were incubated with the mixture of proteins, the 45,000-dalton subunit of G/F was labeled in an ARF-dependent fashion, while no \[^{32}P\] was incorporated into the 41,000-dalton protein. Cholera toxin also catalyzed the incorporation of minor amounts of radioactivity into the 52,000-dalton subunit of G/F, which was present in small amounts in this preparation, and into an unidentified band of approximately 37,000 daltons (Fig. 4, lanes 2 and 3).

The detection of approximately equal amounts of the 41,000-dalton subunit for IAP and a 35,000-dalton protein in the fractions obtained from the heptylamine-Sepharose column suggested that they could be subunits of a single entity. The inability to resolve the 41,000- and 35,000-dalton peptides by a number of chromatographic procedures (ion exchange, high resolution gel filtration, heptylamine-Sepharose, hydroxypatite) was also consistent with this possibility. More definitive evidence that these two peptides are associated was obtained by examination of the hydrodynamic properties of the purified IAP substrate (Table I). These determinations indicate that the substrate for IAP is a molecule with a molecular weight of approximately 80,000 (not corrected for possible binding of cholate). After incubation with Mg\(^{2+}\), ATP, and F\(^-\), IAP substrate activity behaves like a particle with \(M_r = 50,000\). This situation is entirely analogous to that observed with G/F (13, 14).

The preparation of protein used for the hydrodynamic studies consisted predominantly of 41,000- and 35,000-dalton polypeptides (80% of the protein visualized) and contained no G/F activity. Equilibrium binding assays, performed as described by Northup et al. (20), revealed 0.7 mol of \[^{32}S\]GTP\(_y\)S bound per 75,000 g of protein (data not shown). We believe, therefore, that the substrate for IAP contains a guanine nucleotide binding site. After incubation with Mg\(^{2+}\) and \[^{32}S\]GTP\(_y\)S, guanine nucleotide binding activity behaves like a particle with \(M_r = 50,000\) and is hydrodynamically indistinguishable from IAP substrate activity in the presence of Mg\(^{2+}\), ATP, and F\(^-\). Again, this behavior is identical to that shown by G/F (13, 14). We suggest that the substrate for IAP is a dimer of 41,000- and 35,000-dalton subunits and that the dimer dissociates into its component polypeptides in the presence of GTP\(_y\)S or F\(^-\).

We attempted to localize the guanine nucleotide binding site by use of a photolyzable GTP analog, 8-azido\[^{32}P\]GTP. Photoincorporation of \(^{32}P\) was examined with a fraction from the heptylamine-Sepharose column that contained both G/F and IAP substrate activity (Fig. 5). Both the 45,000-dalton subunit of G/F and the 41,000-dalton subunit of the IAP substrate were labeled to a similar extent. Labeling of both polypeptides was blocked by inclusion of unlabeled GTP\(_y\)S, but not by ATP. Little or no label was incorporated into the 35,000-dalton polypeptide. These data indicate that the 41,000-dalton peptide of the IAP substrate contains a site for

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**Fig. 3. Autoradiographic analysis of radioactive products resulting from IAP- or cholera toxin-catalyzed ADP-ribosylation of rabbit liver membranes and purified protein fractions.** Sample proteins were incubated with \[^{32}P\]NAD in the presence of IAP or preactivated cholera toxin as indicated below and as described under "Experimental Procedures." Lane 1, rabbit liver membranes plus 20 \(\mu\)g/ml of IAP; Lane 2, 41,000/35,000-dalton protein fraction; lane 3, 41,000/35,000-dalton protein plus ARF plus 50 \(\mu\)g/ml of cholera toxin; lane 4, 41,000/35,000-dalton protein plus ARF plus 50 \(\mu\)g/ml of cholera toxin; lane 5, 41,000/35,000-dalton protein plus ARF; lane 6, 41,000/35,000-dalton protein + ARF + 20 \(\mu\)g/ml of IAP; lane 7, 41,000/35,000-dalton protein + 20 \(\mu\)g/ml of IAP; lane 8, Coomassie blue stain of 41,000/35,000-dalton protein fraction; lane 9, Coomassie blue stain of G/F; lane 10, G/F + ARF + 50 \(\mu\)g/ml of cholera toxin; lane 11, G/F + 50 \(\mu\)g/ml of cholera toxin; lane 12, G/F + ARF; lane 13, G/F + ARF + 20 \(\mu\)g/ml of IAP; lane 14, G/F + 20 \(\mu\)g/ml of IAP; lane 15, G/F. Labeled bands with lower molecular weights correspond to the A; subunit of cholera toxin (lanes 3, 4, and 11) and to the S, subunit of IAP (lanes 6, 7, 13, and 14).
Substrate for Islet-activating Protein

**Table I**

Hydrodynamic parameters of the substrate for IAP

| Parameter                      | Incubation prior to analysis |
|--------------------------------|-----------------------------|
|                                | None | AMP | GTP-S |
| Stokes radius, (nm)            | 4.43 ± 0.02 | 3.61 ± 0.05 | 3.73 ± 0.05 |
| Sedimentation coefficient, \( s_{20, w} (S) \) | 4.31 ± 0.12 | 3.29 ± 0.17 | 3.21 ± 0.18 |
| Molecular weight \( (M_r) \)   | 82,000 | 51,000 | 51,000 |
| Frictional coefficient, \( f/f_0 \) | 1.42 | 1.35 | 1.40 |

* ATP, Mg**, and F** (see "Experimental Procedures").

- Calculated according to the equation

\[
M_r = \frac{S_{20, w} - 6g_{20, w} \cdot N_A \cdot a}{1 - \frac{1}{2p_{20, w}}}
\]

- Calculated according to the following equation, where \( \delta \) is assumed to be 0.2 g of solvent/g of protein

\[
f/f_0 = a \left[ \frac{4\pi N_A}{3M_r \delta + 5(\eta_{sol})} \right]^{1/3}
\]

binding of guanine nucleotides and they further justify the use of \( ^{[35]S} \)GTP-S to monitor the hydrodynamic properties of the substrate.

**DISCUSSION**

Islet-activating protein catalyzes the specific ADP-ribosylation of a 41,000-dalton protein in rat C6 glial cell membranes, and this modification is correlated with the ability of IAP to modulate adenylate cyclase activity (10, 11). We have identified and partially purified the corresponding substrate for IAP-induced ADP ribosylation from rabbit liver plasma membranes. This partially purified preparation consists predominantly of two polypeptides with apparent molecular weights of 41,000 and 35,000. The 41,000-dalton polypeptide is a substrate for IAP but not for cholera toxin, while the 45,000-dalton subunit of G/F is a substrate for cholera toxin but not for IAP.

ADP-ribosylation of this partially purified protein by IAP is not adventitious. Apparently, a single 41,000-dalton protein is ADP-ribosylated by the toxin in hepatic and C6 membranes. The predominant substrate for IAP in detergent extracts of such membranes has an identical molecular weight and co-purifies with G/F during chromatography on DEAE and Aca34; this chromatography results in a 100-fold purification of G/F (13). Resolution of the IAP substrate from G/F is achieved on heptylamine-Sepharose. Full details of this purification scheme will be described when techniques for quantitation of IAP substrate activity in crude extracts have been refined and when contaminating peptides have been removed.

At a semi-quantitative level, however, the substrate protein in the fraction studied to date accounts for the ADP ribosylation that is observed in membranes.

Evidence suggests that the substrate for IAP is a homodimer of 41,000- and 35,000-dalton polypeptides and that there is striking homology between G/F and the substrate for IAP. (i) Schemes for purification of the two proteins are very similar. (ii) Hydrodynamic characterization of the substrate for IAP indicates that the protein behaves as a species with \( M_r = 80,000 \) in the absence of ligands but as a particle with \( M_r = 50,000 \) in the presence of GTP-S or fluoride. These properties are essentially identical to those observed for G/F (13, 14). (iii) The larger subunits of both G/F and the substrate for ADP-ribosylation by specific toxins; they also contain at least one binding site that is specific for guanine nucleotides. (iv) The smaller subunits of each protein are indistinguishable by sodium dodecyl sulfate-gel electrophoresis. Their functional and chemical relationships are under investigation.

ADP-ribosylation of a 41,000-dalton protein by IAP is known to correlate with the ability of IAP to potentiate the effects of stimulatory hormones on adenylate cyclase and to block attenuation of adenylate cyclase activity by inhibitory hormones (10, 11). It is plausible that the catalytic activity of adenylate cyclase is modulated in a reciprocal fashion by a homologous pair of GTP-binding regulatory proteins, G/F (G) and the IAP substrate (G). Also, intriguing is the fact that transducin, a guanine nucleotide-binding regulatory protein of the rod outer segment that interacts with a light-activated cyclic GMP phosphodiesterase, exhibits striking structural and functional similarities (21, 22). An interesting family of proteins appears to be emerging.

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