Employing [32P]ADP-ribosylation by pertussis toxin, we have identified a G protein that is located in the rough endoplasmic reticulum of canine pancreas and therefore termed G\textsubscript{PER}. Identification of G\textsubscript{PER} is based on the following data. A 41-kDa polypeptide was the only polypeptide that was [32P]ADP-ribosylated by pertussis toxin in pancreas rough microsomes. Guanosine 5'-triphosphate (GTP\textgamma S) and 1 mM ATP, 6 mM MgCl\textsubscript{2}, 10 mM NaF, and 2.5 mM MgCl\textsubscript{2} inhibited ADP-ribosylation of this polypeptide. The [32P]ADP-ribosylated 41-kDa polypeptide was immunoprecipitated by antisera which specifically recognized the C-terminal residues of the \(\alpha\) subunits of G\textsubscript{i} and transducin. It also induced the release of the [32P]ADP-ribosylated 41-kDa polypeptide from rough microsomes in the absence of detergent, unlike ADP-ribosylated \(\alpha\) subunits of plasma membrane-associated G proteins. These data are consistent with an oligomeric nature of G\textsubscript{PER}. The codistribution of G\textsubscript{PER} with an endoplasmic reticulum marker protein during subcellular fractionation and the lack of plasma membrane contamination of the rough microsomal fraction, combined with the isodensity of G\textsubscript{PER} with rough microsomes as well as the identity of G\textsubscript{PER} with "s gravy" microsomes after extraction of rough microsomes with EDTA and 0.5 M KCl, localized G\textsubscript{PER} to the rough endoplasmic reticulum. Preliminary experiments suggest that G\textsubscript{PER} appears not to be involved in translocation of proteins across the rough endoplasmic reticulum membrane.

EXPERIMENTAL PROCEDURES

Membrane Preparation—Rat brain plasma membranes were prepared according to Ref. 9. Dog pancreas RM and EDTA- and KCl-extracted microsomes (EK-RM) were prepared as previously described (10).

ADP-ribosylation by Pertussis Toxin—Activation of PT (0.1 mg/ml) was carried out for 20 min at 37°C in 100 mM Hepes (pH 8.0), 50 mM dithiothreitol, and 2.5 \(\mu\)g/ml bovine serum albumin. Membranes (4-6 mg of protein/ml) were incubated for 1 h at 30°C with 10 \(\mu\)g/ml of the activated toxin and 2.5-5.0 \(\mu\)Ci of [32P]NAD (Du Pont-New England Nuclear, 800-1000 Ci/mmol) in 40 mM Tris-HCl (pH 8.0), 10 mM thymidine, 4 mM ATP, 2.5 mM MgCl\textsubscript{2}, and 25 mM dithiothreitol. The reaction was stopped by chilling on ice.

SDS-Polyacrylamide Gel Electrophoresis—The samples were precipitated for 15 min on ice by addition of an equal volume of 20% trichloroacetic acid, and the pellets were resuspended in sample buffer and analyzed on 8% acrylamide gels according to Laemmli (11). Dried gels were exposed to Kodak XAR-5 film at 70°C.
the antisera against the β subunit of transducin (14) was performed at room temperature for 1 h.

**Protein Determination**—Protein concentration was determined by the method of Bradford (15).

**Sucrose Gradient Centrifugation**—After ADP-ribosylation, the membranes (200 µl) were sedimented in a Beckman Airfuge at 122,000 × g for 10 min and then resuspended in cold buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 6 mM MgCl₂, 0.1% Lubrol. The membranes were incubated for 30 min at 30 °C in the absence or presence of 0.1 mM GTPyS and then layered on top of linear 5-20% sucrose gradients (11.5 ml) made in the same buffer without Lubrol. The gradients were centrifuged at 115,000 × g for 90 min in a Beckman SW 40 rotor at 4 °C. The marker proteins, bovine serum albumin, ovalbumin, soybean trypsin inhibitor, and cytochrome c, were also centrifuged in parallel on separate gradients. 40 fractions were collected from each gradient, each fraction was trichloroacetic acid-precipitated, and the trichloroacetic acid precipitates were analyzed by SDS-PAGE using 12% acrylamide gels.

**Subcellular Fractionation of the Dog Pancreas Homogenate**—The different steps from the homogenization of the pancreas to the isolation of the microsomal membranes were performed as previously described (10) except that the homogenate was centrifuged at 7,000 × g for 20 min. This modification provided the simultaneous pelleting of nuclei and mitochondrial fractions. For subsequent ADP-ribosylation, this pellet was homogenized (Dounce homogenizer, five strokes) in a hypotonic buffer equivalent to that of the supernatant. Equivalent isolations of the microsomal membranes were performed as previously described (17). The membranes were incubated without (control membranes) or with 100 nM ADP-ribosylated, and then resuspended in sample buffer for SDS-PAGE.

**Characterization of a Substrate for PT in the Rough Microsomal Fraction from Dog Pancreas**—Bacterial toxins such as cholera toxin (CT) or pertussis toxin (PT) are known to ADP-ribosylate α subunits of specific G proteins. Thus, the α subunit of transducin is a substrate for ADP-ribosylation by both CT (18) or PT (19), whereas that of Gs is a substrate only for CT (20) and those of G(11) and Ga (21, 22, 23), only for PT.

When an RM fraction from dog pancreas was incubated with PT and [32P]NAD, a single polypeptide that migrated in SDS-PAGE with an apparent molecular mass of 41 kDa was ADP-ribosylated (Fig. 1A, lane 2). When compared to the substrates for PT of a rat brain plasma membrane preparation, the canine pancreatic RM contained a 45 kDa protein that was ADP-ribosylated as described under "Experimental Procedures." TCA-precipitated and then analyzed on a 9% SDS-polyacrylamide gel. A, lane 1, rat brain plasma membranes (40 µg of protein); lane 2, dog pancreas RM (60 µg of protein); lane 3, dog pancreas ER-RM (40 µg of protein). B, dog pancreas RM were preincubated for 30 min at 30 °C with the various effectors and the mixtures were then incubated for ADP-ribosylation. Lane 1, no addition; lane 2, 0.1 mM GDP; lane 3, 0.1 mM GTP; lane 4, 1 µM GTPyS; lane 5, AMF. C, after ADP-ribosylation, rat brain membranes (lanes 1 and 2) or dog pancreas RM (lanes 3 and 4) were immunoprecipitated with nonimmune serum (lanes 1 and 3) or AS6 antiseraum (lanes 2 and 4). M, markers were ovalbumin (45 kDa) and carbonic anhydrase (31 kDa).
tion, namely the 41- and the 39-kDa α subunits of Gi, (21) and G, (22, 23), respectively (Fig. 1A, lane 1), the RM fraction contained only a 41- and not a 39-kDa species. Extraction of the RM fraction by EDTA and high salt did not remove the PT substrate (Fig. 1A, lane 2), suggesting that this protein was not associated with ribosomes and that its attachment to the microsomal membrane did not depend primarily on ionic interactions.

As reported for transducin (19) and G, (24), "activating" ligands like GTPγS and AMP (Fig. 1B, lane 1 versus lanes 4 and 5, respectively) inhibited the ADP-ribosylation by PT, whereas, "stabilizing" ligands like GDP and GTP enhanced it (Fig. 1B, lanes 2 and 3).

To determine whether the ADP-ribosylated polypeptide in the RM fraction is immunologically related to the β subunits of G proteins, we used an antiserum raised against the C-terminal residues of the α subunit of Gi (13). This antiserum has been shown to immunoprecipitate the subunit of both G and transducin, but not G, (13). As expected, of the two ADP-ribosylated α subunits of a rat brain plasma membrane fraction, only the 41-kDa polypeptide corresponding to the α subunit of Gi was immunoprecipitated by this antiserum (Fig. 1C, lane 2). Also specifically immunoprecipitated (nonimmune serum controls are shown in lanes 1 and 3 of C) was the ADP-ribosylated protein of the RM fraction (Fig. 1C, lane 4), demonstrating that this protein is immunologically related to the α subunit of G, and transducin.

We also carried out Western blot analysis (data not shown) of the RM fraction using the anti-Gi α subunit serum (see above) and an anti-transducin β subunit serum. Either serum gave only barely detectable signals which were, however, clearly detectable after partial purification of the ADP-ribosylated protein from the RM fraction.2

Disassembly of the PT Substrate in the Rough Microsome Fraction by GTPγS—To investigate whether the ADP-ribosylated protein in the RM fraction is indeed a GTPγS-dissociable subunit of a complex, aliquots of EDTA- and KCl-extracted RM were ADP-ribosylated, solubilized with Lubrol, incubated in the presence or absence of GTPγS, and analyzed by sucrose gradient centrifugation. The sedimentation rate of the ADP-ribosylated and detergent-solubilized protein incubated with GTPγS was found to be slower than its counterpart incubated in the absence of GTPγS (Fig. 2). Thus, like the α subunits of other G proteins, the microsomal substrate for PT appeared to be part of a complex that is disrupted by ligands like GTPγS.

Only in the case of transducin is the GTPγS-dissociated α subunit also released from the rod outer segment membranes (4). To determine whether, following GTPγS-induced dissociation of the complex, the PT substrate is either released from the microsomal membrane or remains anchored to it, ADP-ribosylated microsomal membranes were incubated in the absence or presence of GTPγS, and membrane-associated material was separated from membrane-released material by differential centrifugation. About half of the PT substrate was found to be released from the membrane as a result of the GTPγS-induced dissociation of the complex (Fig. 3A). An even larger release was observed when ADP-ribosylation and subsequent incubation with GTPγS was carried out at low ionic strength (Fig. 3B). We conclude that, like the α subunit of transducin, the PT substrate in the RM fraction is not independently anchored to the membrane (as may be the case for the α subunits of other G proteins) but is released from the membrane upon ligand-induced dissociation of the complex.

2 S. K. Nigam, unpublished data.
peptides were transferred to nitrocellulose and probed with mono-

to a partial removal of ribosomes from RM during the 1-h

fraction and the pellet fraction and the RM fraction is virtually free

of plasma membrane is likely to be located in the nuclei/mito-
dochondria fraction (Fig. 5, RM, fraction 6). Likewise, Golgi membranes possibly con-
taining G, that serves as substrate for PT, our data (Fig. 5) show that G, of plasma membrane density was not detectable in our RM fraction. Furthermore, the bulk of the dog pancreas plasma membranes is likely to be located in the nuclei/mitochondria pellet fraction and the RM fraction is virtually free of plasma membrane (Fig. 4). On the assumption that the rate of ADP-ribosylation was similar for the PT substrates in the plasma- and microsomal membranes and from the relative distribution of the endoplasmic reticulum (ER) and plasma membrane markers, as well as the PT substrates in the postmitochondrial supernatant- and nuclei/mitochondria fraction, we conclude that the bulk of the PT-catalyzed ADP-

ribosylated α subunits of dog pancreas G proteins is associated with the ER membrane-linked G protein and not with plasma membrane-linked G proteins.

ADP-ribosylation by PT of the Rough Microsome Fraction Does Not Affect Translocation—Because of the reported requiremen of GTP for a productive interaction of the signal sequence of presecretory proteins with microsomal membranes (8), we investigated the effect of PT-catalyzed ADP-

ribosylation of the RM fraction on its capacity to function in translocation of secretory proteins (Fig. 6). The ADP-ribo-
sylated RM fraction yielded a slight inhibition of translation.

<table>
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<th>FRACTION NUMBER</th>
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**Fig. 5. Localization of the PT substrate to microsomal membranes.** Rat brain plasma membranes (upper part) and dog pancreas RM (middle part) or EK-RM (lower part) were ADP-

ribosylated, and the equilibration density of the PT substrate was analyzed by isopycnic centrifugation on linear 31–55% sucrose gra-
dients using a flotation protocol. The figure shows the autoradiograms of the 14 fractions collected from the top (left) to the bottom (right) of each gradient plus a 15th fraction obtained by resuspension of the pellet.

substrate in the RM fraction was indeed associated with the rough microsomal membrane and not with the plasma mem-
brane. Nevertheless, it might be argued that the PT substrate of the RM fraction could represent clathrin-coated vesicles which might cofractionate with RM and which might be involved in the endocytosis and recycling of G proteins from the plasma membrane. However, we consider this unlikely. First, clathrin-coated vesicles are lighter than 55% sucrose (27) and would therefore not be expected to sediment under the experimental conditions used in Fig. 5. Second, ADP-

ribosylation was carried out at pH 8.0 (see “Experimental Procedures”), and ADP-ribosylated RM were placed into heavy sucrose for flotation (Fig. 5); both conditions induce uncoating of clathrin from coated vesicles (28–30); the un-

coated vesicles would be expected to equilibrate at a density similar to that of the brain plasma membrane; no PT sub-

strate was detected at that density in the RM fraction (Fig. 5, RM, fraction 6). Likewise, Golgi membranes possibly con-
taminating our RM fraction are an unlikely source of the PT substrate in our RM fraction because they would be expected to equilibrate at densities even lighter than EK-RM (Fig. 5).

Although dog pancreas plasma membranes most likely con-
tain G, that serves as substrate for PT, our data (Fig. 5) show that G, of plasma membrane density was not detectable in our RM fraction. Furthermore, the bulk of the dog pancreas plasma membrane is likely to be located in the nuclei/mito-
dochondria fraction, and the RM fraction is virtually free of plasma membrane (Fig. 4). On the assumption that the rate of ADP-ribosylation was similar for the PT substrates in the plasma- and microsomal membranes and from the relative distribution of the endoplasmic reticulum (ER) and plasma membrane markers, as well as the PT substrates in the postmitochondrial supernatant- and nuclei/mitochondria fraction, we conclude that the bulk of the PT-catalyzed ADP-

ribosylated α subunits of dog pancreas G proteins is associated with the ER membrane-linked G protein and not with plasma membrane-linked G proteins.

**Fig. 4. Subcellular fractionation of the PT microsomal sub-
strate.** The different subcellular fractions were isolated as described under “Experimental Procedures.” In A, the same volume from each fraction was ADP-ribosylated, trichloroacetic acid-precipitated, and subjected to SDS-PAGE. In B, the samples were not ADP-ribosylated, and after trichloroacetic acid precipitation and SDS-PAGE, the polypeptides were transferred to nitrocellulose and probed with monoclonal antibodies directed against ribophorin I. Lane 1, rat brain membranes; lane 2, dog pancreas homogenate; lane 3, “postmitochondrial” supernatant; lane 4, nuclei/mitochondrial pellet; lane 5, load zone; lane 6, smooth microsomes; lane 7, rough microsomes. In C, transfer was performed as in B (except for lane 1 where no membranes were loaded), but probed with polyclonal sera against γ-glutamyl transpeptidase (separate fractionation experiment). Densitometric analysis revealed that 44% of the PT substrate is recovered in the postmitochondrial supernatant of which 13% are found in the “post-
microsomal” supernatant, 21% in smooth microsomes, and 66% in rough microsomes.
that of the ADP-ribosylated, detergent-solubilized RM, suggesting dissociation of the α-subunit from a complex; and, as demonstrated for the α-subunit of transducin (4), we found a GTPγS-induced release of the ADP-ribosylated 41-kDa polypeptide from RM in the absence of detergent, suggesting that it is not directly linked to the lipid bilayer of RM.

That this protein was indeed a unique GRER and not a plasma membrane-associated G protein (such as Gq) that might have contaminated our RM fraction was shown by cell fractionation and isopinic sucrose gradient centrifugation. Using ribophorin I as a marker for the endoplasmic reticulum, the distribution of GRER during cell fractionation paralleled that of ribophorin I. The distribution of γ-glutamyl transpeptidase in the fractionation confirmed that the RM fraction was essentially free of plasma membranes. In isopinic sucrose gradient centrifugation GRER banded with the RM. Most importantly, stripping of RM by EDTA and 0.5 mM KCl, a procedure that removes many of the peripheral membrane proteins and ribosomes and therefore leads to a characteristic shift of the stripped RM to a lighter density, also resulted in an exactly overlapping density shift of stripped RM and GRER. By comparison, Gq and Go, that are associated with a plasma membrane fraction from rat brain sedimented at a density even lighter than that of the stripped membranes. Taken together, these data clearly ruled out a plasma membrane origin for the G protein in the dog pancreas RM fraction and localized this protein to the RER.

The exocrine acinar cells constitute the bulk of the pancreatic tissue. Estimates based on morphometry suggest that 60% of the membranes of these cells are RER (32). The abundance of the RER in these cells may have facilitated the detection of GRER. Pancreas is likely to also contain Gα that is ADP-ribosylatable by PT. Our cell fractionation data suggest that ADP-ribosylatable GRER is present in pancreas in considerable excess relative to ADP-ribosylatable Gq, reflecting the abundance of RER in these cells. Although little is known about the biosynthesis of G proteins, because of the large excess of ADP-ribosylatable GRER over Gq, it seems unlikely that GRER represents a biosynthetic intermediate of Gq. Moreover, the fact that the α-subunit of GRER is released from the RER membrane points to an important functional difference from the G proteins localized in the plasma membrane.

Although our sedimentation data (Fig. 2) suggest that GRER is a heterotrimeric G protein, our evidence for this is not yet definitive. A partially purified GRER reacted in Western blots with both α subunit- and β subunit-specific antibodies. Definitive identification of GRER as a heterotrimer has to await its purification to homogeneity.

The function of GRER remains to be elucidated. Our preliminary data (Fig. 6) argue against, but do not rule out, an involvement of GRER in protein translocation.

**REFERENCES**