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Pertussis Toxin-Mediated ADP-Ribosylation of Target Proteins in Chinese Hamster Ovary Cells Involves a Vesicle Trafficking Mechanism

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Pertussis toxin (PT)-catalyzed ADP-ribosylation of target proteins in intact Chinese hamster ovary (CHO) cells was evaluated with an in vitro ADP-ribosylation assay. In this assay, a postnuclear supernatant was prepared from CHO cells and used as a source of PT-sensitive target proteins for in vitro [^{32}P]ADP-ribosylation. The postnuclear supernatant contained three proteins that were ADP-ribosylated in vitro, with apparent molecular masses of 50, 45, and 42 kDa. The 42- and 45-kDa proteins were membrane associated, while the 50-kDa protein was soluble. Following PT treatment of CHO cells, the 42- and 45-kDa proteins were not available for in vitro ADP-ribosylation, while the soluble 50-kDa protein remained available for in vitro ADP-ribosylation. The decrease in the availability of the 42- and 45-kDa proteins to in vitro ADP-ribosylation was proportional to the PT concentration and time of incubation with CHO cells. Western immunoblot analysis showed that extracts from PT-treated CHO cells and control CHO cells possessed equivalent amounts of two proteins that were recognized by anti-G_i protein antiserum. The two proteins recognized by anti-G_i protein antiserum from PT-treated cells migrated with higher apparent molecular weights than the two proteins from control cells. This was consistent with the in vivo ADP-ribosylation of the two proteins by PT. NH_4Cl, inhibitors of intracellular trafficking, reduced temperature, and brefeldin A inhibited the ability of PT to ADP-ribosylate the 42- and 45-kDa proteins in vivo. These data implicate a pH-sensitive step and intracellular trafficking system in the in vivo ADP-ribosylation of G_i proteins by PT.

Pertussis toxin (PT; molecular mass, 105,060 Da) is a member of a family of bacterial exotoxins which catalyze the transfer of the ADP-ribose portion of NAD to specific target proteins in eukaryotic cells (47). PT is composed of six noncovalently associated proteins, designated S1 through S5, which are organized in a 1:1:1:2:1 ratio, respectively. PT follows the A:B model for exotoxin structure-function (14). The A component (S1) possesses ADP-ribosyltransferase activity, and the B component (S2 through S5) binds to target cells and delivers S1 to the cytosolic side of the plasma membrane. The receptor-binding region of the B oligomer appears to include S2 and S3, since S2-S4 and S3-S4 heterodimers bind to mammalian cells (21, 35, 43, 47). A glycoprotein with an N-linked oligosaccharide has been implicated as the cell surface receptor for PT (1, 4). The ability of S2 and S3 to penetrate lipid membranes may reflect their ability to facilitate the internalization of S1 (31). Upon entry into the cell, S1 ADP-ribosylates a cysteine residue located near the carboxyl terminus of the α subunit of several heterotrimeric G proteins, including G_i, G_o, and G_t. ADP-ribosylation uncouples signal transduction between G proteins and their G protein-coupled receptors (18).

Hewlett and coworkers (17) showed that PT stimulated the clustering of CHO cells in a dose-dependent process. While this is a sensitive and useful assay, PT-stimulated clustering of CHO cells is temporally distal to the ADP-ribosylation of G_i proteins, which limits the use of this assay in measuring the in vivo ADP-ribosylation of G_i protein.

A direct procedure to measure ADP-ribosylation in intact cells has been described (44). Following the radiolabeling of intracellular NAD with [2-^{3}H]adenine, PT stimulated the radiolabeling of a 41-kDa protein in intact cells. Treatment with PT prior to metabolic labeling of cells blocked subsequent radiolabeling of the 41-kDa protein by PT. Although it is sensitive to PT treatment, this assay did not appear to be amenable to quantitative evaluation of the in vivo ADP-ribosylation of G proteins.

Burns and coworkers (5) showed that PT stimulation of CHO cell clustering required catalytically active PT and implicated a role for the ADP-ribosylation of a 41-kDa protein in CHO cell clustering with an in vitro assay, while Ui described the use of a similar in vitro assay to implicate a 41-kDa PT-sensitive target protein in C6 cells (48). In the present study, an in vitro assay was used to quantitatively evaluate the ability of PT to ADP-ribosylate target proteins in intact CHO cells. In this assay, intact cells were treated with PT to ADP-ribosylate G_i proteins with nonradioactive endogenous NAD. Extracts were prepared from these cells and used as a source of G_i proteins in an in vitro [^{32}P]ADP-ribosylation reaction. Thus, a decrease in the amount of target protein available for in vitro [^{32}P]ADP-ribosylation reflects their in vivo ADP-ribosylation. This assay identified the in vivo ADP-ribosylation of two PT-sensitive target proteins in CHO cells and showed that the in vivo ADP-ribosylation of these two proteins was sensitive to NH_4Cl, reduced temperature, and brefeldin A (BFA).

MATERIALS AND METHODS

Materials. CHO K1 cells were obtained from the American Type Culture Collection (CCL 61). PT was a gift of Rino Rappuoli (Istituto Ricerche Immunobiologiche, Siena, Italy). ^{125}I was purchased from Amersham Corp. Adenylate [^{32}P]phosphate-labeled NAD ([^{32}P]NAD) was purchased from Du Pont-New England Nuclear. Protein molecular weight markers were purchased from Pharmacia-LKB Biotechnology Inc. Rabbit anti-G_α common immunoglobulin G (IgG; catalog no. 371737) was purchased from Calbiochem-Novabiochem (San Diego, Calif.), and rabbit anti-$G_{i\alpha}$ common antiserum (catalog no. 06-190) was

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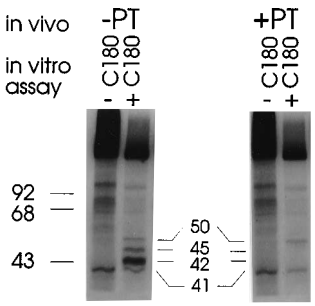

FIG. 1. In vitro ADP-ribosylation of PT-sensitive target proteins in CHO cell extracts. Intact CHO cells were incubated alone (in vivo, −PT) or with 100 pM PT (in vivo, +PT) for 16 h at 37°C. Cells were harvested, washed, and homogenized, and a PNS was prepared. The PNS was incubated in the absence (in vitro assay, −C180) or in the presence of 1.0 μM C180 peptide (in vitro assay, +C180) for 1 h with 0.1 μM [^{32}P]NAD in 100 mM Tris-HCl (pH 7.6)–20 mM DTT. The reaction mixture was subjected to SDS-PAGE (10% acrylamide in a 4 to 6 M urea gradient). The gel was stained with Coomassie blue, dried, and subjected to autoradiography. A photograph of the autoradiogram is shown. The migration of protein molecular size standards (in kilodaltons) is indicated to the left of the autoradiogram. Apparent molecular masses of PT-sensitive target proteins are indicated in kilodaltons between the panels.

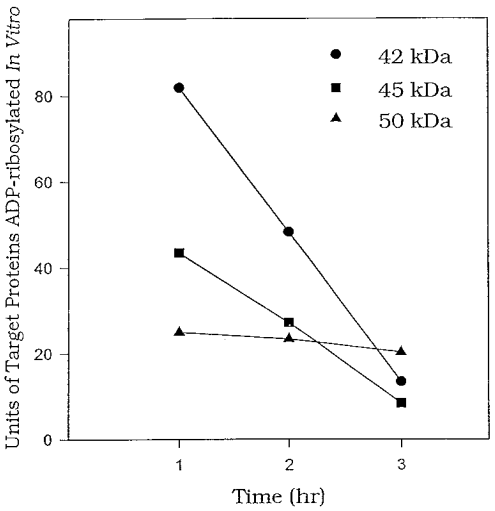

FIG. 2. In vitro ADP-ribosylation of PT-sensitive target proteins following treatment of CHO cells with PT for various time periods. Intact CHO cells were incubated with 100 pM PT for 1, 2, or 3 h at 37°C. Cells were harvested, washed, and homogenized, and a PNS was prepared. The PNS was incubated for 1 h in the presence of 1.0 μM C180 peptide with 0.1 μM [^{32}P]NAD in 100 mM Tris-HCl (pH 7.6)–20 mM DTT. The reaction mixture was subjected to SDS-PAGE (10% acrylamide in a 4 to 6 M urea gradient). The gel was stained with Coomassie blue, dried, and subjected to autoradiography. PT-sensitive target proteins detected on the autoradiogram were quantified with an AMBIS optical imaging system. Arbitrary units were normalized to total protein loaded per lane and plotted against the incubation time. The results of a representative experiment are shown.

purchased from Upstate Biotechnology Inc. (Lake Placid, N.Y.). BFA, Triton X-100, CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate), and NH_4Cl were purchased from Sigma Chemical Company (St. Louis, Mo.). Thesit (catalog no. 836-630) was purchased from Boehringer GmbH (Mannheim, Germany).

Preparation of PNS and cell membrane. CHO cells were cultured to confluency on 15-cm petri dishes at 37°C in a 5% CO_2 atmosphere in Ham's F-12 medium supplemented with 10% newborn calf serum. After the indicated treatment (see text and figure legends for specific treatments), CHO cells were washed twice with 5 ml of ice-cold Dulbecco's phosphate-buffered saline, scraped off the dish into HES buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4], 1 mM EDTA, 255 mM sucrose), and homogenized with a tissue grinder (Kontes). After centrifugation of the homogenate at 3,000 × *g* for 5 min to pellet nuclei and unbroken cells, the postnuclear supernatant (PNS) was used as the source of PT-sensitive target proteins in an in vitro ADP-ribosylation assay. The PNS was also subjected to ultracentrifugation at 128,000 × *g* for 20 min; the membrane fraction was suspended in 1% CHAPS in 25 mM Tris-HCl (pH 7.6) and, along with the soluble fraction, used as a source of PT-sensitive target proteins in the in vitro ADP-ribosylation assay. The membrane fraction was also suspended in sodium dodecyl sulfate (SDS) sample buffer containing β-mercaptoethanol and subjected to immunoblotting.

In vitro ADP-ribosylation assay. The in vitro ADP-ribosyltransferase reaction mix contained, in 24 μl, 0.1 M Tris-HCl (pH 7.6), 20 mM dithiothreitol (DTT), 0.1 μM [^{32}P]NAD (specific activity, 100 Ci/mmol), 5 μl of PNS or the membrane or soluble fraction of the PNS, and 5 μl of 5 μM C180 peptide. Purification of the C180 peptide from *Escherichia coli* has been described (2). The assay was performed at room temperature and terminated after 1 h by the addition of 6 μl of SDS sample buffer containing β-mercaptoethanol and boiling for 5 min at 100°C. Samples were subjected to urea gradient SDS-polyacrylamide gel electrophoresis (PAGE). The gel was dried and exposed to X-ray film. Quantification of autoradiograms was performed with an AMBIS optical imaging system. The arbitrary units obtained were normalized to the amount of total protein loaded on the gel.

Urea gradient SDS-PAGE. Electrophoresis was performed in a slab system (Hoefer Instruments; 0.75 mm by 8.3 cm by 10.2 cm) with Laemmli's running buffer (25) containing a 2-cm stacking gel and a 5-cm separating gel. The stacking gel contained 6.0% acrylamide, 0.2% bisacrylamide, 62.5 mM Tris-HCl (pH 6.9), 0.1% SDS, 0.02% ammonium persulfate, and 0.1% TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine). The separating gel contained 10% acrylamide, 0.35% bisacrylamide, 0.375 M Tris-HCl (pH 8.9), 0.1% SDS, 0.02% ammonium persulfate, and 0.05% TEMED in a linear gradient of 4 M (top) to 6 M (bottom) urea. Electrophoresis was performed at 80 V for 25 min and then at 200 V for 65 min.

Immunoblotting. Confluent CHO cells were incubated alone or with 100 pM PT at 37°C for 3 h prior to the preparation of the PNS. PNS was subjected to ultracentrifugation at 128,000 × *g* for 20 min. The membrane fraction was suspended in SDS sample buffer containing β-mercaptoethanol and boiled at 100°C for 5 min. Approximately 40 μg of total membrane proteins per lane was loaded on urea gradient (4 to 6 M)–SDS–10% polyacrylamide gels. Proteins were separated by electrophoresis as described in the previous paragraph. Proteins were electrophoretically transferred to a nitrocellulose filter at 1.0 A for 2 h at 4°C. The nitrocellulose filter was washed at room temperature for 30 min with solution A (10 mM Tris-HCl [pH 8.0], 2 mM EDTA, 50 mM sodium chloride, 0.1% sodium iodine) containing 1% hemoglobin and then transferred to solution A containing 1% hemoglobin and either rabbit anti-G_α common IgG or rabbit anti-$G_{i\alpha}$ common antiserum overnight at room temperature. The nitrocellulose filter was washed three times (5-min incubations) with solution B (0.1 M Tris-HCl [pH 8.0], 0.2 M sodium chloride) containing 1% Triton X-100 before it was incubated with ^{125}I-protein A in solution A containing 1% hemoglobin at room temperature for 8 h. The filter was washed three times (5-min incubations) with solution B containing 1% Triton X-100, air dried, and exposed to X-ray film.

C180 peptide preparation. The C180 peptide is composed of the N-terminal 180 residues of the S1 subunit of PT. The properties and purification of the C180 peptide from *E. coli* have been described (2).

Protein determination. Protein concentrations were determined by the method of Bradford (3) with a bicinchoninic acid protein assay kit (Pierce Chemical Company, Rockford, Ill.).

RESULTS

In vitro ADP-ribosylation of PT-sensitive target proteins in CHO cell extracts. PNS were prepared from intact CHO cells that were incubated alone or with 100 pM PT at 37°C for 16 h. Microscopic examination showed that the PT-treated cells possessed a clustered cell morphology, while the control cells possessed a typical spindle cell morphology. The PNS was used as a source of target protein in an in vitro ADP-ribosylation reaction by the C180 peptide with [^{32}P]NAD. The PNS from control CHO cells possessed three proteins with apparent molecular masses of 50, 45, and 42 kDa that were specifically radiolabeled by the C180 peptide in vitro (Fig. 1, left panel). The intensity of the in vitro radiolabeling of the 50-, 45-, and 42-kDa proteins by the C180 peptide shown in Fig. 1 was representative of other experiments. In addition, the PNS from control cells possessed a high-molecular-weight radiolabeled band with an apparent molecular mass of >92 kDa and a 41-kDa band that were radiolabeled in the absence of the C180 peptide (Fig. 1, left panel) and considered to represent proteins that were not PT sensitive. Analysis of the PNS from CHO cells treated with PT (Fig. 1, right panel) showed that the

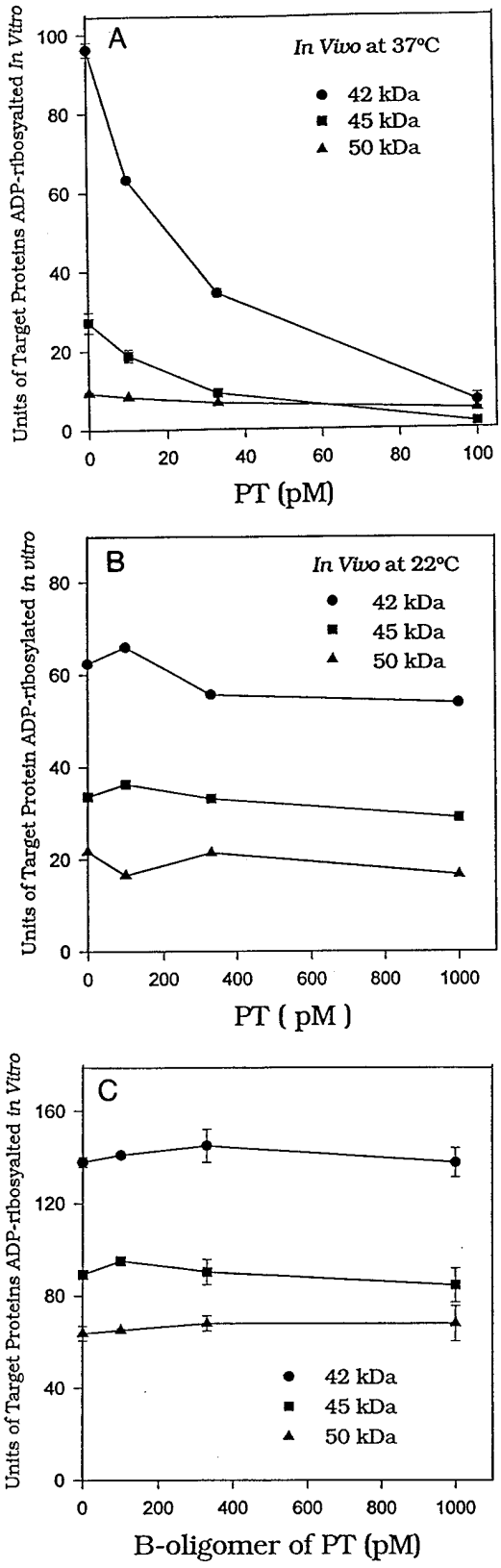

FIG. 3. In vitro ADP-ribosylation of PT-sensitive target proteins in cell extracts following incubation of CHO cells with various concentrations of PT or the B oligomer of PT at 22 or 37°C. Intact CHO cells were incubated with the indicated concentration of PT at 37°C (A) or 22°C (B) or the B oligomer of PT at 37°C (C) for 3 h. Cells were harvested, washed, and homogenized, and a PNS was prepared. The PNS was incubated for 1 h with 1.0 μM C180 peptide with 0.1 μM [^{32}P]NAD in 100 mM Tris-HCl (pH 7.6)–20 mM DTT. The reaction mixture was subjected to SDS-PAGE (10% acrylamide in a 4 to 6 M urea gradient). The gel was stained with Coomassie blue, dried, and subjected to autoradiography. PT-sensitive target proteins that were detected on the autoradiogram were quantified with an AMBIS optical imaging system. Arbitrary units were normalized to total protein loaded per lane and plotted against the concentration of PT or the B oligomer of PT.

intensity of the radiolabel incorporated into the 42- and 45-kDa target proteins had decreased, while the intensity of the radiolabel incorporated into the 50-kDa target protein did not change with respect to the amount of radiolabel incorporated into control cells. The PNS from CHO cells treated with PT also possessed a high-molecular-weight radiolabeled band with an apparent molecular mass of >92 kDa and a 41-kDa band that was radiolabeled in the absence of the C180 peptide (Fig. 1, right panel). A mixing experiment showed that addition of the PNS prepared from PT-treated CHO cells did not inhibit the ADP-ribosylation of transducin by the C180 peptide. This indicated that the PNS from PT-treated cells did not contain an apparent inhibitor of the ADP-ribosylation reaction (data not shown). Other experiments showed that the S1 subunit of PT ADP-ribosylated the same PT-sensitive target proteins in the PNS of CHO cells as were ADP-ribosylated by the C180 peptide in vitro (data not shown). These data correlated the inability to ADP-ribosylate the 42- and 45-kDa target proteins in vitro with the ability of PT to stimulate a clustering morphology in CHO cells.

In vitro ADP-ribosylation of PT-sensitive target proteins in CHO cell PNS following incubation with PT or the B oligomer of PT at 37°C. Analysis of the PNS from intact CHO cells incubated with 100 pM PT at 37°C for 1, 2, and 3 h showed a time-dependent decrease in the availability of the 42- and 45-kDa proteins to serve as target proteins in the in vitro ADP-ribosylation assay, while a constant amount of the 50-kDa protein was available for in vitro ADP-ribosylation throughout the time course (Fig. 2).

Incubation of intact CHO cells with increasing amounts of PT (between 0 and 100 pM) at 37°C for 3 h resulted in a dose-dependent decrease in the availability of the 42- and 45-kDa proteins to serve as PT-sensitive target proteins in the in vitro ADP-ribosylation assay (Fig. 3A). In contrast, the incorporation of radiolabel into the 50-kDa target protein in the PNS in the in vitro ADP-ribosylation assay was constant with increasing concentrations of PT. Analysis of the PNS from intact CHO cells that had been incubated with increasing amounts of the B oligomer of PT (between 0 and 1 nM) at 37°C for 3 h showed that the availability of the 42-, 45-, and 50-kDa proteins in the PNS to serve as PT-sensitive target proteins in the in vitro ADP-ribosylation assay remained constant (Fig. 3C). This indicated that the reduction in the availability of the 42- and 45-kDa proteins for in vitro ADP-ribosylation required ADP-ribosyltransferase activity when PT was incubated with intact CHO cells.

Distribution of PT-sensitive target proteins in the PNS of CHO cells. Since the sensitivity of the 50-kDa target protein to in vivo ADP-ribosylation was different from that of the 42- and 45-kDa target proteins, we examined their subcellular localization. PNS from PT-treated or control CHO cells were subjected to ultracentrifugation to obtain a particulate (membrane) fraction and a soluble (cytosolic) fraction. In vitro ADP-ribosylation of the control cell PNS showed that >85% of the 42- and 45-kDa PT-sensitive target proteins was in the particulate fraction of the PNS, while approximately 70% of

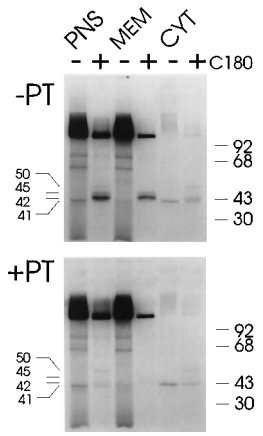

FIG. 4. Distribution of PT-sensitive target proteins in CHO cell extracts following ultracentrifugation. Intact CHO cells were incubated alone (upper panel, −PT) or with 100 pM PT (lower panel, +PT) for 3 h at 37°C. Cells were harvested, washed, and homogenized, and a PNS was prepared. The PNS was subjected to ultracentrifugation at 124,000 × *g* for 20 min at 4°C to separate soluble (CYT) and particulate (MEM) fractions. The PNS, MEM, and CYT fractions were incubated for 1 h alone (−) or in the presence of 1.0 μM C180 peptide (+) with 0.1 μM [^{32}P]NAD in 100 mM Tris-HCl (pH 7.6)–20 mM DTT. The reaction mixture was subjected to SDS-PAGE (10% acrylamide in a 4 to 6 M urea gradient). The gel was stained with Coomassie blue, dried, and subjected to autoradiography. A photograph of an autoradiogram is shown. The migration of protein molecular size standards (in kilodaltons) is indicated to the right of the autoradiograms. Apparent molecular masses of PT-sensitive target proteins (in kilodaltons) are indicated to the left of the autoradiograms.

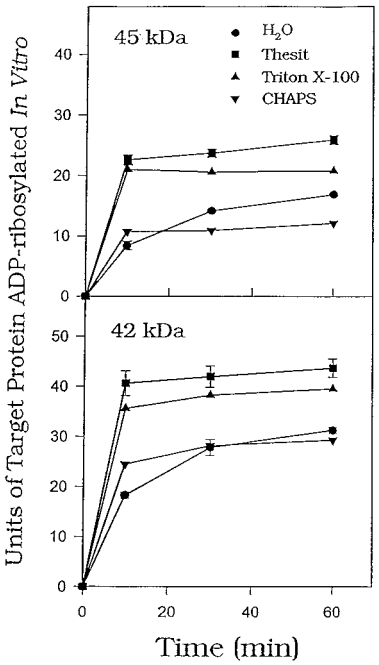

FIG. 5. In vitro ADP-ribosylation of PT-sensitive target proteins in the presence of detergents. Confluent CHO cells were harvested, washed, and homogenized, and a PNS was prepared. The PNS was incubated for 10, 30, or 60 min in the presence of 0.1% Thesit, 1% Triton X-100, 1% CHAPS, or no detergent (H_2O) and 1.0 μM C180 peptide with 0.1 μM [^{32}P]NAD in 100 mM Tris-HCl (pH 7.6)–20 mM DTT. The reaction mixture was subjected to SDS-PAGE (10% acrylamide in a 4 to 6 M urea gradient). The gel was stained with Coomassie blue, dried, and subjected to autoradiography. PT-sensitive target proteins detected on the autoradiogram were quantified with an AMBIS optical imaging system. Arbitrary units were normalized to total protein loaded and plotted against the incubation time.

the 50-kDa PT-sensitive target protein was in the cytosolic fraction of the PNS (Fig. 4). The membrane fraction of the PNS from PT-treated cells showed a decreased availability of the 42- and 45-kDa proteins for in vitro ADP-ribosylation, while the 50-kDa protein in the cytosolic fraction of the PNS from cells treated with PT remained available for in vitro ADP-ribosylation. Microscopic examination showed that treatment of CHO cells with 100 pM PT at 37°C for 3 h did not stimulate a cell clustering response. Thus, the in vivo ADP-ribosylation of the 42- and 45-kDa target proteins by PT precedes the PT-stimulated clustering of CHO cells.

In vitro ADP-ribosylation of PT-sensitive target proteins in the presence of detergents. Since PT-sensitive target proteins could partition into micelles during the preparation of the PNS and therefore not be available for in vitro ADP-ribosylation, a question arose whether the in vitro ADP-ribosylation assay was detecting only a minor population of PT-sensitive target proteins in the PNS. Experiments determined whether the addition of detergents to the PNS increased the amount of PT-sensitive target proteins that could be ADP-ribosylated in the in vitro ADP-ribosylation reaction. For the 42-kDa target protein, addition of either 0.1% Thesit or 1.0% Triton X-100 to the PNS from control CHO cells increased the amount of PT-sensitive target protein that could be ADP-ribosylated in vitro by approximately 30%, while the addition of 1.0% CHAPS did not affect the amount of target protein that could be ADP-ribosylated in vitro in the absence of detergents (Fig. 5). Similar results were obtained for the 45-kDa target protein (Fig. 5). These data indicate that the majority of PT-sensitive target proteins in the PNS were accessible for in vitro ADP-ribosylation.

Immunoblot of G proteins from control and PT-treated CHO cells. Roerig and coworkers (39) reported that ADP-ribosylation caused a decrease in the mobility of the α subunit of G proteins during urea-SDS-PAGE. This observation allowed the direct determination of whether the nonavailability of PT-sensitive target proteins to in vitro ADP-ribosylation was due to in vivo ADP-ribosylation or a depletion of the G protein pool upon PT treatment. Immunoblot analysis of membrane fractions from the PNS of CHO cells treated with PT or control CHO cells identified two proteins that reacted with rabbit anti-$G_{i\alpha}$ common antiserum (Fig. 6B). While the amount of immunoreactivity of the two $G_{i\alpha}$-reactive proteins in the PT-treated extracts was similar to that of control cells, the electrophoretic mobility of the two $G_{i\alpha}$-reactive proteins in the PT-treated extracts decreased with respect to the mobility of the two $G_{i\alpha}$-reactive proteins detected in the control CHO cell extract.

Immunoblot analysis of anti-G_α common IgG in extracts from the PNS of CHO cells treated with PT or control CHO cells showed a more complex pattern of immunoreactive proteins than the immunoblot probed with anti-$G_{i\alpha}$ common antiserum (Fig. 6A). In extracts from control cells, one major and one minor protein band were detected, which corresponded to the two immunoreactive proteins recognized by the anti-$G_{i\alpha}$ common antiserum. With respect to the immunoreactivity observed in control cell extracts, extracts from PT-treated cells showed similar amounts of the major and minor immunoreactive proteins which corresponded to the G_i-reactive proteins, and the majority of the major immunoreactive band and the minor immunoreactive band showed decreased electrophoretic mobility. Similar amounts of a 50-kDa protein that was also recognized by anti-G_α common IgG were detected and observed to possess similar electrophoretic mobilities in extracts

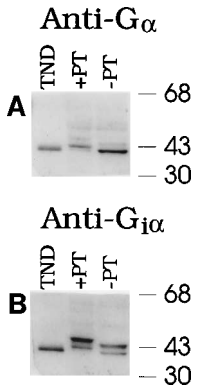

FIG. 6. Immunoblot of G proteins from control and PT-treated CHO cells. Intact CHO cells were incubated alone (−PT) or with 100 pM (+PT) at 37°C for 3 h. Cells were harvested, washed, and homogenized, and a PNS was prepared. The PNS was subjected to ultracentrifugation at 128,000 × *g* for 20 min at 4°C to obtain the membrane fraction. Membrane fractions were suspended in SDS sample buffer containing β-mercaptoethanol and boiled at 100°C for 5 min. Approximately 40 μg of total membrane proteins per lane was subjected to urea gradient (4 to 6M)–SDS–10% PAGE. Proteins transferred to nitrocellulose filters were incubated with rabbit anti-G_α common IgG (A) or rabbit anti-$G_{i\alpha}$ common antiserum (B) followed by ^{125}I-protein A. An autoradiogram of the immunoblot is shown. Purified transducin (TND) was used as a control in the leftmost lane. The migration of protein molecular size standards (in kilodaltons) is indicated to the right of the autoradiogram.

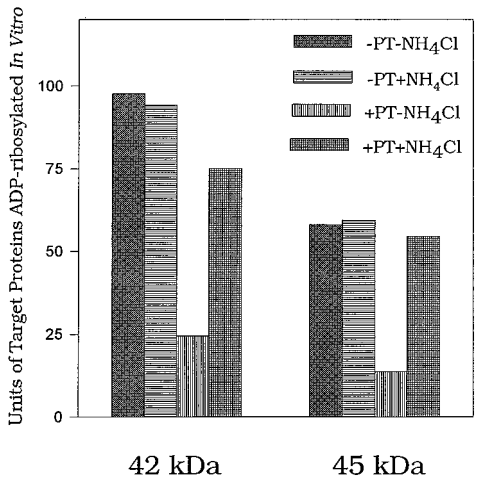

FIG. 7. In vitro ADP-ribosylation of PT-sensitive target proteins following treatment of CHO cells with PT and NH_4Cl. Intact CHO cells were incubated alone (−PT−NH_4Cl), with 100 pM PT (+PT−NH_4Cl), with 10 mM NH_4Cl (−PT+NH_4Cl), or with 100 pM PT and 10 mM NH_4Cl (+PT+NH_4Cl) for 3 h at 37°C. Cells were harvested, washed, and homogenized, and a PNS was prepared. The PNS was incubated for 1 h in the presence of 1.0 μM C180 peptide with 0.1 μM [^{32}P]NAD in 100 mM Tris-HCl (pH 7.6)–20 mM DTT. The reaction mixture was subjected to SDS-PAGE (10% acrylamide in a 4 to 6 M urea gradient). The gel was stained with Coomassie blue, dried, and subjected to autoradiography. Quantification was performed on the autoradiogram with an AMBIS optical imaging system. Arbitrary units were normalized to total protein loaded in each lane. A representative experiment is shown.

from control and PT-treated cells. These data support the hypothesis that the decrease in availability of the 42- and 45-kDa target proteins in PT-treated extracts of CHO cells to in vitro ADP-ribosylation was due to their in vivo ADP-ribosylation and not due to a depletion of G_i protein pools. These data also showed that the apparent molecular masses assigned to the 42- and 45-kDa target proteins were greater than their actual molecular masses.

In vitro ADP-ribosylation of PT-sensitive target proteins in PNS following incubation of CHO cells at 22°C. Temperature-sensitive steps have been reported in the intoxication of cells by modeccin, cholera toxin, and ricin (9, 26, 40). The PNS from PT-treated CHO cells that had been incubated at 22°C for 3 h with up to 1 nM PT, a concentration 10-fold higher than was necessary to ADP-ribosylate the 42- and 45-kDa proteins in vivo at 37°C, possessed the same profile of radiolabeled PT-sensitive target proteins as the PNS from control CHO cells that had been incubated at 22°C for 3 h (Fig. 3B). This indicated that at 22°C, PT failed to ADP-ribosylate the 42- and 45-kDa PT-sensitive target proteins in vivo. The inhibition of the in vivo ADP-ribosylation of target proteins by PT was reversible, since shifting CHO cells from 22 to 37°C reestablished the ability of PT to ADP-ribosylate the 42- and 45-kDa target proteins in vivo (data not shown). It seemed unlikely that the inhibitory effect that we observed was due to the inhibition of the in vivo ADP-ribosylation reaction at 22°C, since there was not a substantial inhibition of the in vitro ADP-ribosylation of target proteins at room temperature. These results indicated that a temperature-sensitive step was required for the in vivo ADP-ribosylation of the 42- and 45-kDa PT-sensitive target proteins.

In vitro ADP-ribosylation of PT-sensitive target proteins following incubation of CHO cells with PT and NH_4Cl. To determine whether pH influenced the in vivo ADP-ribosylation of PT-sensitive target proteins, the effect of NH_4Cl, an acidotropic agent, on the in vivo ADP-ribosylation of target proteins by PT was determined. PNS from PT-treated CHO cells that had been incubated in the presence of 10 mM NH_4Cl for 3 h possessed greater amounts of the 42- and 45-kDa target proteins for in vitro ADP-ribosylation than did extracts from cells treated with PT alone (Fig. 7). This indicated that NH_4Cl inhibited the in vivo ADP-ribosylation of the 42- and 45-kDa proteins by PT. Incubation of CHO cells with 10 mM NH_4Cl alone at 37°C for 3 h did not change the amount of PT-sensitive target proteins available for in vitro ADP-ribosylation (Fig. 7). Control experiments showed that 10 mM NH_4Cl did not inhibit the in vitro ADP-ribosylation reaction (data not shown).

The NH_4Cl inhibition of in vivo ADP-ribosylation of target proteins by PT was transient. The 42- and 45-kDa target proteins in the PNS from CHO cells incubated with 100 pM PT in the absence or presence of 10 mM NH_4Cl at 37°C for 16 h were not available for in vitro ADP-ribosylation. Microscopic examination showed that 16 h of incubation with 100 pM PT yielded a clustered CHO cell morphology independent of the presence of 10 mM NH_4Cl (data not shown). These results implicate a transient ammonium chloride-sensitive but not -dependent step in the intoxication of CHO cells by PT.

Control experiments showed that the addition of 1.0% Triton X-100 to the in vitro ADP-ribosylation reaction mix did not change the profile shown in Fig. 7 (data not shown). These data indicated that the nonavailability of the 42- and 45-kDa target proteins to in vitro ADP-ribosylation was not due to a redistribution of the target proteins in vivo which prevented their in vitro ADP-ribosylation.

In vitro ADP-ribosylation of PT-sensitive target proteins following treatment of CHO cells with PT and BFA. Recently, BFA, a fungal antibiotic, has been shown to inhibit the cytotoxicity of abrin, ricin, modeccin, *Pseudomonas* exotoxin A, and cholera toxin (11, 19, 34, 41, 49). The PNS from PT-treated CHO cells that had been incubated in the presence of 1 μg of BFA per ml at 37°C for 3 h possessed greater amounts of the 42- and 45-kDa target proteins available for in vitro ADP-ribosylation than did extracts from cells treated with PT

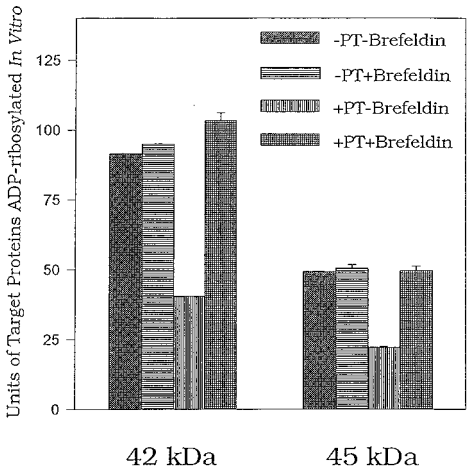

FIG. 8. In vitro ADP-ribosylation of PT-sensitive target proteins following treatment of CHO cells with PT and BFA. Intact CHO cells were incubated alone (−PT−BFA), with 100 pM PT (+PT−BFA), with 1 μg of BFA per ml (−PT+BFA) or with 100 pM PT and 1 μg of BFA per ml (+PT+BFA) for 3 h at 37°C. Cells were harvested, washed, and homogenized, and a PNS was prepared. The PNS was incubated for 1 h in the presence of 1.0 μM C180 peptide with 0.1 μM [^{32}P]NAD in 100 mM Tris-HCl (pH 7.6)–20 mM DTT. The reaction mixture was subjected to SDS-PAGE (10% acrylamide in a 4 to 6 M urea gradient). The gel was stained with Coomassie blue, dried, and subjected to autoradiography. Quantification was performed on the autoradiogram with an AMBIS optical imaging system. Arbitrary units were normalized to total protein loaded in each lane.

alone (Fig. 8). This indicated that BFA inhibited the in vivo ADP-ribosylation of the 42- and 45-kDa proteins by PT. Incubation of CHO cells with 1 μg of BFA per ml alone at 37°C for 3 h did not decrease the availability of the 42- and 45-kDa target proteins for in vitro ADP-ribosylation (Fig. 8). Control experiments also showed that BFA did not inhibit the ADP-ribosylation of transducin by the C180 peptide (data not shown). These results implicate a Golgi-mediated intracellular trafficking pathway for the in vivo ADP-ribosylation of target proteins by PT.

An experiment was performed to determine whether BFA influenced the interaction of PT with cell surface receptors. CHO cells were incubated with 400 pM PT in the absence or presence of 1 μg of BFA per ml at 4°C for 30 min. Cells were washed to remove unbound PT and then incubated at 37°C for 4 h. PNS from cells treated with PT in the absence or presence of BFA possessed the same amount of 42- and 45-kDa target proteins available in the in vitro ADP-ribosylation assay (data not shown). This indicated that BFA did not interfere with the interaction between PT and cell surface receptors. Similar experiments also showed that 10 mM NH_4Cl did not interfere with the interaction between PT and cell surface receptors.

DISCUSSION

Members of the family of bacterial ADP-ribosylating exotoxins interfere with host cell function by enzymatically modifying intracellular target proteins. While the NAD-binding domains of several of these exotoxins have been shown to possess a common structure and a conserved active-site glutamic acid (45), the regions of the exotoxins that deliver the catalytic domain to the intracellular target protein share little apparent primary amino acid homology or three-dimensional structure. Delivery of the catalytic domain to intracellular target proteins requires the exotoxin to bind cell surface receptors, translocate the catalytic subunit across the cell membrane, and process the catalytic domain into an enzymatically active form. One challenge to exotoxin research is to correlate the observed in vitro biochemical properties with the mechanism utilized to intoxicate cells in vivo.

Immunologic techniques have shown that CHO cells possess both G_{i2} and G_{i3} proteins (13). These studies did not determine the subcellular distribution of G_{i2} and G_{i3}. In the present study, an in vitro ADP-ribosylation assay was used to measure the in vivo ADP-ribosylation of PT-sensitive target proteins in CHO cells. Two PT-sensitive target proteins with apparent molecular masses of 42 and 45 kDa (in the ADP-ribosylated form) were detected. Immunoblot analysis showed that PT-treated and control CHO cells possessed similar amounts of two proteins that reacted with anti-$G_{i\alpha}$ antiserum and had decreased electrophoretic mobilities following PT treatment. This indicated that the decreased availability of the 42- and 45-kDa target proteins in PT-treated extracts for in vitro ADP-ribosylation was due to their in vivo ADP-ribosylation and not due to a depletion of G_i proteins. Other studies have reported that ADP-ribosylated G_i proteins show decreased electrophoretic mobilities with respect to non-ADP-ribosylated G_i proteins (39) and that PT treatment of cells does not reduce G_i protein pools (7). Other investigators have used a similar in vitro assay to identify PT-sensitive target proteins in several cell lines (5, 48).

Hewlett and coworkers showed that PT stimulated a morphological response that resulted in CHO cell clustering (17). Although the molecular mechanism responsible for this response has not been defined, Burns and coworkers (5) showed that PT and not its subunits was required to elicit this response and implicated a 41-kDa protein as the target for ADP-ribosylation. Since this assay is sensitive and also requires the ADP-ribosyltransferase activity of PT, it is a useful assay for measuring the cytotoxic capacity of PT and mutated forms of PT. The in vitro assay for ADP-ribosylation used in the present study showed a quantitative correlation between the in vivo ADP-ribosylation of two PT-sensitive target proteins in CHO cells and PT stimulation of CHO cell clustering. Our studies also showed that the in vivo ADP-ribosylation occurred prior to the detection of CHO cell clustering. While the in vitro measurement of the in vivo ADP-ribosylation of PT-sensitive target proteins is more labor intensive than the measurement of PT stimulation of CHO cell clustering, it is a direct measure of in vivo ADP-ribosylation. The two assays complement each other; the in vitro ADP-ribosylation assay measures the ability of PT to ADP-ribosylate specific G proteins in vivo, while PT stimulation of CHO cell clustering represents a physiological consequence of the ADP-ribosylation of G proteins. Future studies will correlate the in vivo ADP-ribosylation of the two subsets of G_i proteins with PT stimulation of CHO cell clustering.

The mechanisms used by several bacterial ADP-ribosylating exotoxins to modify target proteins in vivo have begun to be defined. Diphtheria toxin binds sensitive cells via the precursor of a heparin-binding epidermal growth factor-like growth factor (33) and enters cells through receptor-mediated endocytosis (22). The catalytic A domain is translocated across the endosomal membrane via a pH-dependent conformational change within the B domain (32). Agents that prevent endosome acidification, such as NH_4Cl, inhibit the cytotoxic activity of diphtheria toxin. Cholera toxin binds sensitive cells via ganglioside GM_1 (30). Although it was originally thought to directly penetrate the plasma membrane to ADP-ribosylate the G_s protein, recent data have implicated a vesicle system in the delivery of cholera toxin to target protein. A consensus endoplasmic reticulum retention sequence, KDEL, within the car-

boxyl terminus of the cholera toxin A subunit may contribute to the targeting of cholera toxin to G proteins in a processing and/or delivery capacity. The ability of BFA and reduced temperatures to inhibit cholera toxin stimulation of cyclic AMP levels (26, 38) and morphological changes of CHO cells (8) also support a role for a vesicle-mediated trafficking mechanism. *Pseudomonas* exotoxin A binds cells via the α_2-macroglobulin receptor/low-density lipoprotein receptor-related protein (23) and enters cells by receptor-mediated endocytosis (36, 37). The ability of chloroquine, monensin, and BFA to protect cells from exotoxin A-mediated cytotoxicity (12), as well as the presence of a KDEL-like sequence, REDLK, at the carboxyl terminus, implicates an intracellular trafficking pathway that includes the Golgi and endoplasmic reticulum. Ogata et al. (36) have also shown that proteolytic processing is required for exotoxin A-mediated cytotoxicity.

With respect to diphtheria toxin, cholera toxin, and exotoxin A, less is known about the mechanism that PT uses to intoxicate cells. Early models proposed that following the binding of PT to cell surface receptors, the S1 subunit was translocated across the plasma membrane and released from the B oligomer and ADP-ribosylated PT-sensitive G_i proteins (20). This model was based on several in vitro observations which showed that in the presence of detergents, ATP stimulated the release of S1 from PT (6), S1 had greater affinity for lipid bilayers than B oligomer (16), and NH_4Cl did not inhibit PT stimulation of CHO cell clustering (16). The molecular basis for the in vitro activation of PT by ATP has recently been defined. ATP stimulated a reversible conformational change within PT which converted S1 to a catalytically active form without concomitant release of S1 from the B oligomer (24).

Plant and bacterial toxins appear to utilize receptor-mediated vesicle trafficking pathways to gain access to intracellular target proteins. However, these toxins often respond differently to inhibitors of the vesicle trafficking pathways, which suggests that these toxins may be processed at a unique step(s) in the trafficking pathway. While NH_4Cl inhibits the in vivo activity of both diphtheria toxin and PT, the magnitude of this inhibition differs. NH_4Cl caused a transient delay in the ability of PT to ADP-ribosylate two target proteins. This may reflect the inability of NH_4Cl to modify intracellular pH over an extended period of time or indicate that PT is delivered or processed via a step that was stimulated by, but not dependent on, an acidic environment. In contrast, NH_4Cl has been shown to inhibit the action of diphtheria toxin following an overnight incubation (16). Thus, it appears that the entry of diphtheria toxin into cells requires an acidic environment to deliver the catalytic domain across the endosomal vesicle and that there is a temporal window for the successful translocation of diphtheria toxin across the endosomal membrane during the cycling of the endosomal vesicle, presumably prior to endosome-lysosome fusion.

BFA blocked the in vivo ADP-ribosylation of two target proteins by PT at a step downstream of the binding of PT to cell surface receptors. In contrast, intracellular trafficking of diphtheria toxin beyond the endosome was not required, since BFA did not protect Vero cells from the action of diphtheria toxin (29). BFA did protect cultured cells from cholera toxin, exotoxin A, modeccin, and ricin, which, like PT, implicated the involvement of the Golgi apparatus in their intoxication process (8, 34, 38, 49). Thus, the response to BFA has differentiated the productive intracellular trafficking of diphtheria toxin from that of other ADP-ribosylating toxins, including exotoxin A, cholera toxin, and PT, as well as non-ADP-ribosylating toxins like modeccin and ricin.

Integration of the data obtained in the present study with

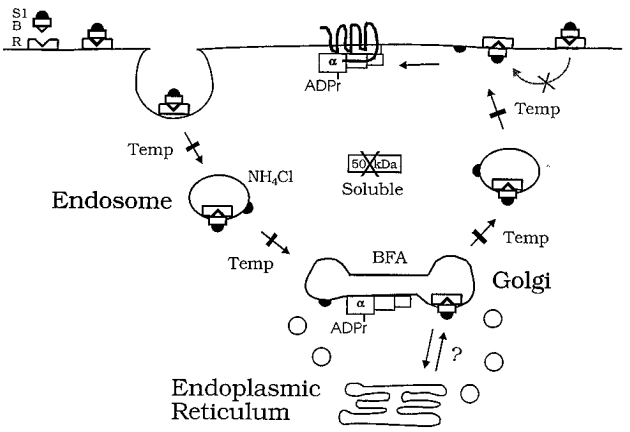

FIG. 9. Schematic model of the intoxication process of eucaryotic cells by PT. PT, composed of S1 and the B oligomer, binds cell surface receptors (R) and undergoes receptor-mediated endocytosis. Reduced temperature (Temp) has been shown to inhibit intracellular vesicle fusion and formation at multiple steps. PT passes through a pH-sensitive step (NH_4Cl) and requires a functional Golgi appparatus (BFA). PT does not appear to enter the cytoplasm, since a 50-kDa target protein that is available for in vitro ADP-ribosylation (ADPr) is not ADP-ribosylated in vivo.

studies by others allows the development of a model for the entry and intoxication of eukaryotic cells by PT (Fig. 9). PT binds to cell surface glycolipids or a glycoprotein receptor via the B oligomer and enters cells by an endocytic vesicle-mediated process which is pH sensitive but not dependent. S1 within the holotoxin or released from the B oligomer remains associated with the cell membrane and trafficks through a BFA- and temperature-sensitive pathway to ADP-ribosylate Golgi- and plasma membrane-associated G_i proteins. Since a KDEL-like sequence is not apparent within the primary amino acid sequence of PT, the involvement of the endoplasmic reticulum in this trafficking pathway is not proposed. The inability to detect the in vivo ADP-ribosylation of the soluble 50-kDa target protein in CHO cells suggests that PT remains membrane associated in vivo. Prior to the ADP-ribosylation of PT-sensitive G proteins, S1 is activated by ATP and reduced glutathione at an undetermined step in the pathway. The inhibition of PT-mediated in vivo ADP-ribosylation by NH_4Cl, reduced temperature, and BFA is consistent with intracellular trafficking of PT rather than direct penetration of PT through the plasma membrane. While NH_4Cl has been proposed to interfere with the acidification of the endosome and BFA has been proposed to interfere with the Golgi apparatus, it is difficult to resolve where the temperature-sensitive step in the PT-mediated ADP-ribosylation of target protein occurs. Reduced temperature has been shown to affect selected steps in the intracellular vesicular pathway, including endosome-endosome fusion (27), fusion between endocytic vesicles and lysosomes (10, 40), transport between the endoplasmic reticulum and Golgi stacks (28, 42), and transport through the trans-Golgi network (15). An alternative interpertation of these data is that a factor that is required for the processing and activation of PT is trafficked via an intracellular vesicle pathway that is sensitive to NH_4Cl, reduced temperature, and BFA. Since PT has been shown to ADP-ribosylate Golgi-associated G proteins (46), it appears more likely that it is PT that is delivered via an intracellular vesicular trafficking mechanism into the cell. Consistent with the present study, Gray et al. have recently reported that BFA inhibits the ability of PT to ADP-ribosylate target G proteins in T cells (14a).

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