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Research Article

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Glucose- and GTP-dependent Stimulation of the Carboxyl Methylation of CDC42 in Rodent and Human Pancreatic Islets and Pure β Cells

Evidence for an Essential Role of GTP-binding Proteins in Nutrient-induced Insulin Secretion

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Abstract

Several GTP-binding proteins (G-proteins) undergo posttranslational modifications (isoprenylation and carboxyl methylation) in pancreatic β cells. Herein, two of these were identified as CDC42 and rap 1, using Western blotting and immunoprecipitation. Confocal microscopic data indicated that CDC42 is localized only in islet endocrine cells but not in acinar cells of the pancreas. CDC42 undergoes a guanine nucleotide-specific membrane association and carboxyl methylation in normal rat islets, human islets, and pure β (HIT or INS-1) cells. GTPγS-dependent carboxyl methylation of a 23-kD protein was also demonstrable in secretory granule fractions from normal islets or β cells. AFC (a specific inhibitor of prenyl-cysteine carboxyl methyl transferases) blocked the carboxyl methylation of CDC42 in five types of insulin-secreting cells, without blocking GTPγS-induced translocation, implying that methylation is a consequence (not a cause) of transfer to membrane sites. High glucose (but not a depolarizing concentration of K^+) induced the carboxyl methylation of CDC42 in intact cells, as assessed after specific immunoprecipitation. This effect was abrogated by GTP depletion using mycophenolic acid and was restored upon GTP repletion by coprovision of guanosine. In contrast, although rap 1 was also carboxyl methylated, it was not translocated to the particulate fraction by GTPγS; furthermore, its methylation was also stimulated by 40 mM K^+ (suggesting a role which is not specific to nutrient stimulation). AFC also impeded nutrient-induced (but not K^+-induced) insulin secretion from islets and β cells under static or perifusion conditions, whereas an inactive structural analogue of AFC failed to inhibit insulin release. These effects were reproduced not only by *S*-adenosylhomocysteine (another methylation inhibitor), but also by GTP depletion. Thus, the glucose- and GTP-dependent carboxyl methylation of G-proteins such as CDC42 is an obligate step in the stimulus–secretion coupling of nutrient-induced insulin secretion, but not in the exocytotic event itself. Furthermore, AFC blocked glucose-activated phosphoinositide turnover, which may provide a partial biochemical explanation for its effect on secretion, and implies that certain G-proteins must be carboxyl methylated for their interaction with signaling effector molecules, a step which can be regulated by intracellular availability of GTP. (*J. Clin. Invest.* 1996. 98:540–555.) Key words: pancreatic β cell • insulin secretion • GTP-binding proteins • CDC42 • carboxyl methylation

Introduction

It is well established that glucose induces insulin release by the generation of intracellular second messengers (1). Recently, using specific inhibitors of guanosine triphosphate (GTP) synthesis, we identified a permissive role for GTP as one of the modulators of physiologic insulin secretion (2, 3). However, the exact sites of action of GTP in pancreatic β cells have not been identified thus far. One possible site could involve GTP-binding proteins (G-proteins),[1] which were found to be enriched in the secretory granule fraction of normal rat islets (4–7); this localization would seem to place G-proteins in a strategic location to modulate stimulus–secretion coupling.

Low molecular weight G-proteins play regulatory roles in protein sorting and trafficking, and in the vectorial transport of secretory vesicles or granules in several eukaryotic cells as well as in yeast (8). Many of these G-proteins undergo a series of posttranslational modification reactions at their carboxyl termini, such as isoprenylation and carboxyl methylation, which increase their hydrophobicity and hence their membrane association. Unlike isoprenylation, carboxyl methylation reactions are reversible and subject to short-term regulation (8–11). Thus, carboxyl methylation is a prime candidate step for acute regulation by agonists promoting rapid cell activation; evidence for this formulation has been obtained recently using

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1. *Abbreviations used in this paper:* AFC, *N*-acetyl-*S-trans,trans*-farnesyl-L-cysteine; AGC, *N*-acetyl-*S-trans*-geranyl-L-cysteine; ARF, ADP-ribosylation factor; G-proteins, GTP-binding protein; GDI, GDP-dissociation inhibitor; MPA, mycophenolic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol monophosphate; PIP_2, phosphatidylinositol bisphosphate; SAM, *S*-adenosyl methionine.

PC-12 cells and neutrophils (12, 13). However, it is still uncertain whether the methylation of key G-proteins directly promotes their translocation to the membrane fraction, as suggested by Philips et al. (13) and Backlund (14), or whether the converse is true, as suggested by recent studies of Bokoch et al. (15) and others (16–18).

We reported recently that normal rat pancreatic islets contain at least five isoprenylated G-proteins (20–27 kD). At least two of these proteins ($\sim$ 20–23 kD) are also carboxyl methylated (19). The current studies were carried out, first, to identify the carboxyl methylated proteins in normal rat and human islets as well as pure β cell lines (and their subcellular fractions), and, second, to explore the possible dependence of their carboxyl methylation and membrane-association on GTP availability (both endogenous and exogenous) in both broken and intact cells. The current study focused particularly on CDC42, a low molecular weight G-protein (8, 14, 20, 21) and calcium-binding protein involved in cytoskeletal protein organization and budding of vesicles in secretory cells (8, 10, 21); these functions might be relevant to secretory granule biogenesis and exocytotic secretion. Recent studies (22), including our own (5, 7), have identified CDC42 in normal islets and in transformed β cells by immunoblotting; a 23-kD protein comigrated with authentic CDC42 on SDS-PAGE (19). Lastly, we examined whether the carboxyl methylation of G-proteins could be activated by stimulators of insulin release and, if so, whether the concomitant secretion could be decreased by blockade of carboxyl methylation.

Methods

Materials

S-adenosyl-L-[methyl-^{3}H]methionine (73 Ci/mmol), L-[methyl-^{3}H]methionine (72 Ci/mmol), and *myo*-[2-^{3}H (*N*)]-inositol (20.5 Ci/mmol) were from New England Nuclear (Boston, MA). *N*-acetyl-*S-trans,trans*-farnesyl cysteine (AFC) and *N*-acetyl-*S-trans*-geranyl-L-cysteine (AGC) were generously provided by Dr. Jeffry Stock (Princeton University, Princeton, NJ) or purchased from BIOMOL Research Labs Inc. (Plymouth Meeting, PA) or Cayman Chemical Co. Inc. (Ann Arbor, MI). Monoclonal antisera for CDC42 and purified CDC42 were gifts from Dr. Tony Evans (Onyx Pharmaceuticals, Richmond, CA). In some studies, affinity-purified rabbit polyclonal antiserum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) raised against a peptide corresponding to amino acid residues 167–183 mapping within the carboxyl-terminal domain of human CDC42Hs was used. An affinity-purified polyclonal antibody corresponding to residues 121–137 of rap 1 protein was obtained from Santa Cruz Biotechnology, Inc. ATPγS, App(NH)p, GTP, and GTPγS were purchased from Boehringer Mannheim (Indianapolis, IN). *Myo*-inositol, mycophenolic acid, mannoheptulose, 3-O-methyl glucose, guanine, guanosine, cycloheximide, actinomycin D, and protein A–Agarose were purchased from Sigma Immunochemicals (St. Louis, MO). In some studies, protein A–Agarose purchased from Santa Cruz Biotechnology, Inc. was also used. Sinefungin was a gift from Lilly Research Laboratories (Indianapolis, IN). *S*-adenosyl homocysteine and 3-deazaadenosine were purchased from Southern Research Laboratories (Birmingham, AL).

Insulin-secreting cells

Intact pancreatic islets were isolated from male Sprague-Dawley rats as described previously (2–6) and were hand-picked under a stereomicroscope twice to avoid contamination by acinar cells. Human islets (from a total of five donors) were generously provided by Dr. David Scharp (Washington University Medical School, St. Louis, MO). HIT-T15 cells (passage #72) were kindly provided by Dr. Hui-Jian Zhang and Dr. Paul Robertson (University of Minnesota Medical School, Minneapolis, MN). INS-1 cells (passages #65–76), a mercaptoethanol-sensitive pure β cell line (23), were provided by Dr. Claes Wollheim (University of Geneva, Geneva, Switzerland). Details of culture conditions and the insulin-secretory capacity of HIT and INS-1 cell lines, in response to various secretagogues, have been described previously (23–25). For the carboxyl methylation studies, human islets, rat islets, INS-1 cells, or HIT cells were homogenized in 230 mM mannitol, 70 mM sucrose, and 5 mM Hepes, pH 7.4, containing 1 mM DTT and 2.5 μg/ml each of leupeptin and pepstatin (as described in references 4–7).

Isolation of subcellular fractions from normal rat islets or insulinoma cells

In experiments involving identification of carboxyl methylated proteins in the soluble or particulate fractions, homogenates were subjected to a single centrifugation at 105,000 *g* for 60 min to separate the total particulate and soluble fractions (5, 26, 27). For studies involving quantitation of the carboxyl methylation of endogenous 23-kD protein in the secretory granules, the latter were isolated by a differential centrifugation method described previously (4, 5). Purity of this fraction was evaluated by marker enzyme analysis as described previously (4, 5). Furthermore, the purity of this fraction was also examined by electron microscopy; these data indicated that this preparation contained $>$ 90% β cell granules with $<$ 10% of contaminating heavy mitochondria (4, 5). Purified secretory granule fractions from insulinoma (RIN) cells were kindly provided by Dr. Chris Rhodes (Joslin Diabetes Center, Boston, MA). Details of the purity of this fraction were described elsewhere (28).

Protein prenyl-cysteine carboxyl methylation assay

In cell free preparations. Protein carboxyl methylation assays were carried out at 37°C for different time intervals as described in the text (in a total volume of 100 μl) in homogenates (25–50 μg protein) or secretory granules (8–10 μg protein) or cytosol (80 μg protein) using 50 mM sodium phosphate buffer, pH 6.8, containing 1 mM EGTA and 100 μCi/ml (7 μM) *S*-adenosyl-L-[methyl-^{3}H]methionine (SAM) as a methyl donor, as described by us recently (19). GTPγS and other reagents (e.g., AFC, *S*-adenosyl homocysteine, or sinefungin) were present in their respective concentrations, as indicated in the text. The reaction was started by the addition of SAM and was terminated by the addition of SDS-PAGE sample buffer; labeled proteins were separated on SDS-PAGE (12% acrylamide). Degree of labeling was quantitated either by fluorography of dried gels (previously soaked in Fluoro-Hance autoradiography enhancer, Research Products International Corp., Mount Prospect, IL, or EN^{3}HANCE, NEN-DuPont, Boston, MA) or by vapor-phase equilibration assay (see below). The intensity of the labeling of individual protein bands was quantitated by densitometry of the bands using a Zeineh soft laser scanning/video densitometer (Biomed Instruments, Fullerton, CA) coupled to an IBM PC-compatible computer equipped with software to calculate the individual peak areas (5, 19).

In intact normal islets. Intact islets (200–250 islets per group) were incubated in an isotonic Krebs/Ringer bicarbonate medium in a metabolic incubator (at 37°C) for a period of 2–4 h in the presence of [methyl-^{3}H]methionine (40–100 μCi/ml). Cycloheximide (5 μM) and actinomycin D (2 μM) were added to each tube an hour before the addition of labeled methionine in order to inhibit synthetic incorporation of labeled methionine into new proteins (19). Preincubation of islets with methionine was necessary to pre-label the endogenous SAM pools which form the substrate for methylation reactions (29). After the prelabeling, cells were washed once with an isotonic medium to remove the unincorporated label, and various modulators were added and the incubation was continued for additional time periods (0–2 min) as described in the text. The reaction was quickly terminated by adding ice-cold TCA (10% final). After incubation on ice for 10 min, tubes were centrifuged in an Eppendorf centrifuge at 8,000 rpm for 5 min at 4°C. (The recovery of β cell protein in TCA pellets was $>$ 96%; n = 23 determinations.) Protein pellets were re-

constituted in 100 μl of 100 mM Tris-HCl, pH 8.0, and labeled proteins were separated by SDS-PAGE and identified by fluorography as described above. Degree of carboxyl methylation of 23-kD protein was quantitated by base-labile methanol release (see below). Alternatively, agonist-induced stimulation of the carboxyl methylation of CDC42 and rap 1 was also quantitated in intact HIT cells. For this purpose, intact HIT cells (30–40 × 10^6 cells) were prelabeled with [^{3}H]methionine (40–100 μCi/ml; 2 ml total volume) at 37°C for 60 min in the presence of 0.1 mM glucose. After this, cells were washed (×2) with an isotonic medium and agonist-induced stimulation of the carboxyl methylation of 23-kD protein was quantitated as described above.

To address the regulatory role(s) of endogenous GTP on the carboxyl methylation of CDC42, normal rat islets (350 per group) were cultured at 37°C for 18 h in the presence of diluents, mycophenolic acid (MPA; 25 μg/ml) alone, or MPA with guanine (75 μM). Under these conditions, MPA reduces GTP levels by > 80% (and GTP/GDP ratio by > 60%) in isolated islets; coprovision of guanine or guanosine completely reverses the reduction in GTP (or GTP/GDP ratio) elicited by MPA (2, 3). After this, islets were washed twice with Krebs-Ringer medium consisting of BSA (0.1%) and glucose (11.1 mM) and were labeled with [^{3}H]methionine (50 μCi/ml; 1 ml total volume) for 2 h at 37°C. The labeling was terminated by addition of ice-cold Krebs-Ringer medium. After removal of the medium, islets were homogenized in the homogenization medium; their proteins were separated by SDS-PAGE and the carboxyl methylation of 23-kD protein was quantitated by vapor-phase equilibration assay, as described below.

In studies addressing the effects of GTP depletion on agonist-induced carboxyl methylation of CDC42 in β cells, intact HIT cells (20 × 10^6 cells) were incubated with either diluent alone, MPA (1 μg/ml) alone, or MPA (1 μg/ml) plus guanosine (500 μM) for 6 h at 37°C. Under these conditions, MPA treatment resulted in > 80% reduction in endogenous GTP levels and > 75% reduction in GTP/GDP ratio and concomitant nutrient-induced insulin secretion. Coprovision of guanosine completely restored GTP, GTP/GDP ratio, and nutrient-induced insulin secretion in HIT cells (Meredith, M., G. Li, and S.A. Metz, unpublished observations). After incubation, cells were washed (×2) with an isotonic medium, prelabeled with [^{3}H]methionine, and exposed to various agonists as described above. CDC42 was immunoprecipitated from control, MPA-treated, and MPA plus guanosine–treated cells as described below.

Vapor-phase equilibration (base-labile [^{3}H]methanol release) assay

The α-carboxyl methyl groups on prenyl-cysteine residues of modified G-proteins are base-labile (9, 30, 31). To demonstrate that such sites are specifically modified in insulin-secreting cells, methyl esters were quantified by vapor-phase equilibration assay as described previously (9, 19). Briefly, individual lanes of dried gels were cut into 3- or 5-mm slices and were placed in 1.5-ml Eppendorf centrifuge tubes (without caps) containing 300–500 μl of 1 NaOH. Tubes were placed in 20-ml scintillation vials containing 5 ml of scintillation fluid (Ultima Gold; Packard Instrument Co., Meriden, CT). The vials were then capped and left at 37°C overnight to maximize the base-catalyzed release of [^{3}H]methanol due to hydrolysis of methyl esters. After incubation, tubes were gently removed from the vials and the sides of the tubes were rinsed (into the vials) with an additional 2 ml of scintillant and the radioactivity was determined by scintillation spectrometry.

Immunoprecipitation of CDC42 or rap 1

Carboxyl methylated CDC42 or rap 1 were immunoprecipitated by incubating (0.01%) SDS-solubilized islet proteins with either preimmune serum, anti-CDC42 (1:500), or anti-rap (1:500) at 4°C for 4 h followed by an additional 10–12-h incubation with protein A–Agarose (16 mg/ml) as previously described (26). After washing (×2) of the pellets with 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, agarose-associated proteins were separated by SDS-PAGE and the degree of carboxyl methylation was quantitated either by vapor-phase equilibration assay or by autoradiography. Under these conditions, 74% (n = 2 determinations) of authentic CDC42, which had been carboxyl methylated by enzymes provided by addition of HIT cell homogenates, was recovered in the immunoprecipitate.

Guanine nucleotide-dependent association of CDC42 with the particulate fraction

This was examined by Western blot method using an antiserum directed against the 167–183-amino acid residues of CDC42. To address the GTP-dependent effects on CDC42 translocation to the particulate fraction, it was desirable to deplete intact islets or HIT cells of endogenous GTP. This was achieved by dialyzing homogenates (using a microdialyzer, 1 kD cut off; MRA, Clearwater, FL) against 20 mM Tris-HCl, pH 7.4, containing 1 mM DTT for 10 h at 4°C with multiple changes of the buffer (26). Islet, HIT cell, or INS-1 cell homogenates (100–200 μg protein) were incubated in the presence (and absence) of GTPγS (10–100 μM) or GTP (1 mM) and [^{3}H]SAM (100 μCi/ml) for 37°C for different time intervals as described in the text. To examine if GTP alone can affect translocation of CDC42 independent of its effects on carboxyl methylation, islet or HIT cell homogenates were incubated in the presence of GTPγS (100 μM) and AFC (100 μM) in the absence of SAM for different time intervals as described in the text. After incubation, the total particulate and soluble fractions were isolated by centrifugation at 105,000 g for 90 min. Proteins from these fractions were transferred onto a nitrocellulose membrane after SDS-PAGE and the membranes were then incubated with antiserum directed against CDC42 (1:500–750 dilution) or preimmune serum for 15 h at room temperature with gentle shaking. Immune complexes were identified by a chemiluminescence method using alkaline phosphatase coupled to anti–rabbit IgG (Immune-Lite II; Bio Rad, Hercules, CA). A similar approach was used recently by several investigators in studies involving GTP-dependent translocation of G-proteins (e.g., *rac*2) from the soluble to membrane fraction (13, 15, 32, 33). In experiments involving studies of GTPγS effects on the carboxyl methylation of CDC42, protein bands from each lane corresponding to authentic CDC42 were excised from the SDS-gel and/or nitrocellulose membrane and the carboxyl methylation of CDC42 was quantitated by base-labile methanol release or by densitometry.

Quantitation of farnesyl cysteine methyltransferase activity in insulin-secreting cells

INS-1 cells were disrupted by sonication (2 × 10 s at 4°C) in a homogenization buffer consisting of 20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 250 mM sucrose, 1 mM DTT, and protease inhibitors such as leupeptin (10 μg/ml), pepstatin (2 μg/ml), aprotinin (4 μg/ml each), and PMSF (10 μM). The homogenates were centrifuged at 105,000 *g* for 60 min to separate the total particulate and soluble fractions, and the pellets were reconstituted in the homogenization buffer (∼ 1 mg/ml). The activity of farnesylcysteine carboxyl methyltransferase activity was measured using AFC as a substrate; AFC is esterified at the carboxyl terminus in the presence of [^{3}H]SAM. Additional experimental details of quantitation of farnesylcysteine methyl transferase activity in insulin-secreting cells are provided elsewhere (34).

Localization of CDC42 in endocrine cells by immunocytochemistry and confocal microscopy of whole pancreas and isolated islets

Pancreatic tissue was obtained from adult Sprague-Dawley rats. The animals were deeply anesthetized with chloral hydrate (350 mg/kg; intraperitoneally) and then subjected to a transcardial perfusion with Ca^{2+}-free Tyrode's solution followed by a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 6.9. After fixation, the perfusion continued with 500 ml of 10% sucrose phosphate buffer solution, pH 7.2, for 20–30 min. The pancreas was then rapidly dissected out and transferred to 10% sucrose

buffer solution for 4 h before sectioning. Islets were isolated (35) from adult rats and were fixed immediately in 4% paraformaldehyde for 30 min at room temperature. The islets were thoroughly washed with PBS before applying the primary antiserum (diluted in PBS/0.3% Triton). The islets were incubated in the presence of the primary antibody overnight at 4°C and then washed (×6) with PBS, incubated for an additional 3 h with 10% normal donkey serum, washed for 2 h with three changes of PBS, and then incubated with secondary antibodies overnight at 4°C. For the double labeling experiments, the procedures were similar except that the cells were treated with successive application of the primary antibodies and then incubated with combined application of the secondary antibodies. Tissue processing for sections was similar to that used for intact isolated islets. Experiments were replicated from four to six times. At least 20 islets were examined for each experiment and 15–20 optical sections were examined for each islet.

Primary antibodies used in these studies were: mouse monoclonal antiinsulin, antiglucagon, antisomatostatin (all in 1:100 dilutions; Novo Bio-Labs, Danbury, CT). Rabbit anti-CDC42 (1:100 dilution) was from Santa Cruz Biotechnology, Inc. Secondary antibodies used in these studies were cyanine 3.18–labeled donkey anti–rabbit IgG (1:400 dilution) and fluorescein-labeled donkey anti–mouse IgG (1:150 dilution). These were obtained from Jackson ImmunoResearch Laboratories, (West Grove, PA). The immunostained specimens were examined with a Bio Rad MRC-1000 confocal microscope equipped with a krypton/argon laser (Bio Rad Life Sciences Group, Hercules, CA). For the double labeling experiments, images were pseudo-colored either red or green and merged using Confocal Assistant software written by T.C. Brelje (Department of Cell Biology and Neuroanatomy, University of Minnesota, Minneapolis, MN). In merged images, the cells appear either red or green, or in the case of colocalization, they appear yellow. Final image processing was done using Adobe Photoshop and printed using a Fuji Pictrography 3000 digital printer (Tokyo, Japan). Details of these procedures have been reported previously (36, 37).

Effects of AFC on glucose-activated phosphoinositide turnover in normal rat islets

Phosphoinositide turnover was assayed using modifications of the method of Zawalich and Zawalich (38). Normal rat islets (groups of 100) were cultured overnight in RPMI 1640 medium containing 10% fetal calf serum, 11.1 mM glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM Hepes, pH 7.4, and [^{3}H]*myo*-inositol (25 μCi/ml). The next morning, the culture medium was replaced with an isotonic Krebs-Ringer medium consisting of 0.2% bovine serum albumin and 4.4 mM glucose, and [^{3}H]*myo*-inositol (25 μCi/ml). Islets were labeled for an additional 2 h in a 95% O_2 and 5% CO_2 medium. This second labeling step (in the presence of a lower glucose concentration) was included in order to label any glucose-insensitive inositol pools in islets, as well as to revert any possible noxious effects of the culture in high glucose concentrations. Under these conditions, phosphatidylinositol bisphosphate and phosphatidylinositol monophosphate are labeled to apparent isotopic steady state (39). After labeling, islets were transferred into each of the four perifusion chambers. The islets were perifused with Krebs-Ringer bicarbonate buffer containing 1 mM *myo*-inositol and 3.3 mM glucose (gassed with 95% O_2/5% CO_2) for 50 min. This was followed by an additional perifusion for 45 min with media containing 16.7 mM glucose. Where AFC (100 μM) or AGC (100 μM) effects were studied, BSA was excluded from the incubation medium (19, 30). The flow rate was adjusted to 1 ml/min and the effluent samples were collected every 2 min. The perifusate was analyzed for [^{3}H]inositol, using a previously described method (38, 39). At the end of the incubation, the islets (with the nylon filter) were transferred to polyethylene centrifuge tubes and phospholipids were extracted as described previously (39). The upper (aqueous) and the lower (organic) phases were separated by centrifugation and the organic phase (100 μl) was counted by scintillation spectrometry. The remainder of the organic phase was gassed with argon and stored at −30°C until used for quantitation of inositol containing phospholipids by TLC (see below).

To separate inositol-containing phospholipid substrates, silica gel plates (LK6D, 250 μm layer, Whatman, Madstone, United Kingdom) were exposed to 1.2% potassium oxalate as described previously (40). The organic phase of the islet extracts was spotted on TLC plates. The plates were then developed in a solvent system consisting of methanol:chloroform:ammonium hydroxide:water (100:70:15:25; vol/vol). The relevant areas corresponding to authentic, labeled phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP), and phosphatidylinositol-4,5- bisphosphate (PIP_2) (American Radiolabeled Chemicals, Inc., St. Louis, MO) were identified by autoradiography. Radioactivity in each of these spots was quantitated by scintillation spectrometry.

Insulin release under static or perifusion conditions

Insulin release from isolated islets was quantitated by batch-type static incubations or by perifusion of islets with test solutions (2, 3). Static incubations (in the presence of test compounds or appropriate diluents) were carried out for different time intervals as described in the text. Albumin was excluded from tubes containing AFC or AGC because it binds these agents, thereby reducing their effective concentrations in the medium (19). For perifusion experiments, islets were placed into each of two chambers (100 islets per chamber) and were perifused in parallel as described previously (2). Insulin was quantitated by radioimmunoassay (2, 3, 19). Test agents (100 μM each of AFC or AGC) had no effect on the insulin radioimmunoassay. Furthermore, recovery of insulin (secreted in response to stimulation by 16.7 mM glucose) was not significantly different in the absence or presence (0.2%) of BSA ($P = 0.177$; degrees of freedom [df] = 8), indicating that removal of BSA did not induce significant adherence of insulin to the tubing. To assess insulin content, islets were extracted overnight in 1 ml of acid alcohol (77% ethanol, 22% water, 1% 12 N HCl; vol/vol) at 4°C, the supernatant was removed, diluted (1:10) in PBS to a final dilution of 1:20, and assayed by radioimmunoassay.

Other methods

Protein concentration was quantified colorimetrically using a dye-binding method described by Bradford (41).

Results

Carboxyl methylation of a 23 kD protein(s) in insulin-secreting cells: Guanine nucleotide effects in broken-cell preparations. The carboxyl methylation of an endogenous 23-kD protein was stimulated by GTPγS in homogenates of rat islets (244% of basal), human islets (200%), and HIT cells (351%; Fig. 1). Inclusion of AFC (100 μM), a competitive substrate for protein prenyl methyl transferase, markedly inhibited the GTPγS-dependent carboxyl methylation of this protein in these cell types (Fig. 1). Furthermore, in INS-1 cell homogenates, GTPγS (10 μM) stimulated carboxyl methylation of 23-kD protein (+249%); it was inhibited by 100 μM AFC (−88%; additional data not shown). Inclusion of authentic CDC42 in the assay resulted in significant methylation of this protein by all three preparations tested (Fig. 1). GTPγS (10 μM) also stimulated the carboxyl methylation of exogenous CDC42 using rat islets (600% of basal), human islets (162%), or HIT cells (331%). AFC (100 μM) also inhibited GTPγS-stimulated methylation of authentic exogenous CDC42 by 80–98% (Fig. 1). In contrast, AGC (100 μM), which is neither a substrate for, nor an inhibitor of, purified rodent methyl transferases (30), failed to inhibit the GTPγS-stimulated methylation of either endogenous 23-kD protein or of purified CDC42 (additional

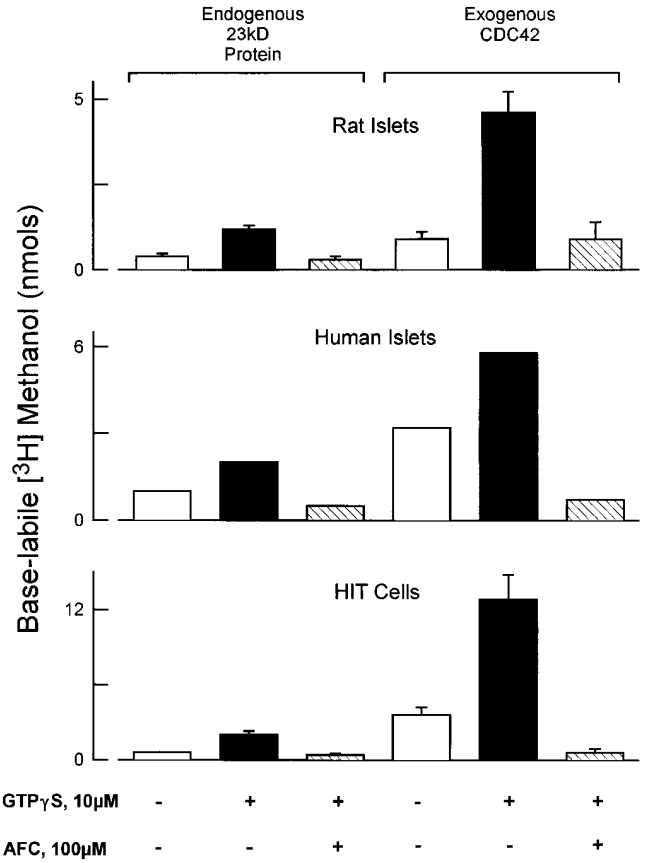

Figure 1. GTPγS stimulates the carboxyl methylation of an endogenous 23-kD protein, as well as of exogenous authentic CDC42 in insulin-secreting cells. Carboxyl methylation was carried out as described in Methods using [^{3}H]SAM (100 μCi/ml; 7 μM) as a methyl donor in the presence or absence of GTPγS (10 μM) and/or AFC (100 μM). Purified CDC42 (1.08 μg) was also present as indicated in the figure. Proteins were separated on SDS-PAGE (12% acrylamide) and the degree of methylation of these proteins was quantitated by base-labile methanol release. Data are mean±SEM of 7–10 experiments using rat islet and/or HIT cell homogenates. Data are from a single experiment using human islet homogenates.

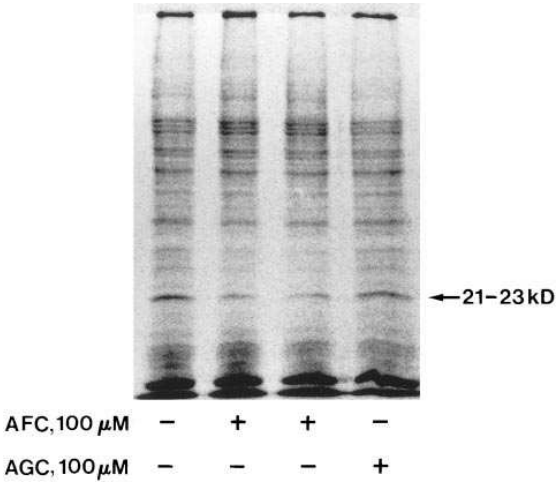

Figure 2. An autofluorograph depicting the inhibition of the carboxyl methylation of a 21–23-kD protein by AFC in intact pancreatic islets. Normal rat islets (groups of 200–250) were preincubated in an isotonic medium in a Krebs-Ringer bicarbonate medium (at 37°C for 4 h) with [^{3}H-methyl]methionine (50 μCi/ml) to label endogenous SAM pools (19). Cycloheximide (5 μM) and actinomycin D (2 μM) were added 1 h before provision of labeled methionine in order to inhibit the synthetic incorporation of [^{3}H]methionine into new proteins. Labeling was carried out for 4 h followed by a 30-min incubation in the presence or absence of either AFC or AGC (100 μM each). Labeled proteins were separated by SDS-PAGE (12% acrylamide) and identified by autofluorography. Results are representative of three independent experiments with similar results.

data not shown). Other inhibitors of protein methylation such as *S*-adenosyl homocysteine or sinefungin (34, 42) also reduced (by 80–90%; additional data not shown) the GTPγS-stimulated carboxyl methylation of the 23-kD protein in normal rat islets or INS-1 cells.

Further studies indicated that the methylation of authentic CDC42 was stimulated by GTPγS in a concentration-dependent manner. One-half of maximal stimulation of carboxyl methylation was demonstrable at GTPγS concentrations of ~ 400 nM and maximal effect occurred between 10 and 100 μM (not shown). In contrast, GTPγS did not stimulate the methylation of the presumptive γ subunit(s) of trimeric G-proteins, migrating at ~ 6–7 kD (43). GTP (500 μM) also stimulated the methylation of CDC42, albeit to a lesser degree (+77%) possibly reflecting GTP hydrolysis by islet endogenous GTPases. Neither ATPγS nor App(NH)p stimulated the methylation of the endogenous 23-kD protein or of exogenous CDC42, suggesting that this effect is guanine nucleotide specific. Fluoroaluminate (25–50 μM) failed to stimulate the carboxyl methylation of exogenous CDC42 (n = 2 experiments; additional data not shown).

Carboxyl methylation of 23-kD protein in intact islets: effects of AFC. To increase the functional relevance of these biochemical events, these studies were extended to intact rat islets in which endogenous SAM pools were pre-labeled with [^{3}H-methyl]methionine (19, 29). Several proteins incorporated [^{3}H]methionine (Fig. 2) even in the presence of the protein synthesis inhibitors cycloheximide and actinomycin D. However, the labeling of only one of these bands (~ 21–23 kD) was inhibited by (each of two different preparations of) AFC (Fig. 2). AGC (100 μM), an inactive analogue of AFC, failed to inhibit the labeling of this protein, suggesting that the 21–23-kD protein may be similar to the one monitored in the five cell-free preparations. Densitometric analyses of the 21–23-kD protein band indicated that AFC inhibited labeling by 50±3% (n = 6 determinations); these data were confirmed by vapor-phase equilibration assay (additional data not shown).

Carboxyl methylation of 23-kD protein in islet subcellular fractions. We also observed the carboxyl methylation of the 23-kD protein in the secretory granule fraction obtained from normal rat islets (Fig. 3). Marker enzyme analyses and electron microscopy data (4, 5) indicated that this fraction is comprised of > 90% β-granules with < 10% contaminating heavy mitochondria. As shown in Fig. 3, GTPγS stimulated the carboxyl methylation of the 23-kD protein in secretory granules by 7.7 fold; this effect was completely inhibited by AFC (100 μM). We also observed a 6.8-fold stimulation by GTPγS of the carboxyl methylation of the 23-kD protein in secretory granules derived from clonal pure β (rat insulinoma) cells. Furthermore, since we observed very little contamination of these fractions by cytosol (as assessed by lactate dehydrogenase activity; references 4 and 5), these data suggest that GTPγS stim-

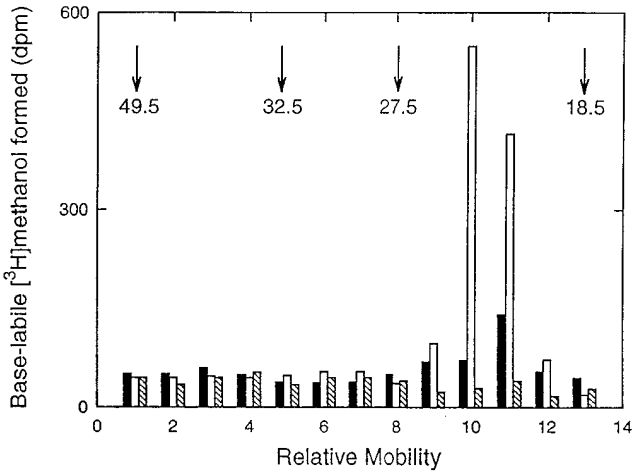

Figure 3. GTPγS stimulates the carboxyl methylation of an endogenous 23-kD protein in the secretory granule fraction of normal rat islets. Carboxyl methylation assay was carried out at 37°C for 60 min as described under Methods, using secretory granule protein (8–10 μg protein) and [^{3}H]SAM (7 μM) in the presence or absence of GTPγS (10 μM). AFC (100 μM) was also present as indicated. Labeled proteins were separated by SDS-PAGE (12% acrylamide) and the degree of methylation was quantitated in gel slices by vapor-phase equilibration assay. Relative mobilities on SDS gels of authentic prestained molecular weight markers are indicated by arrows. Similar data were obtained using secretory granule fraction from pure β (insulinoma) cells (see Results). ■, Control; □, + GTPγS; ▨, + GTPγS + AFC.

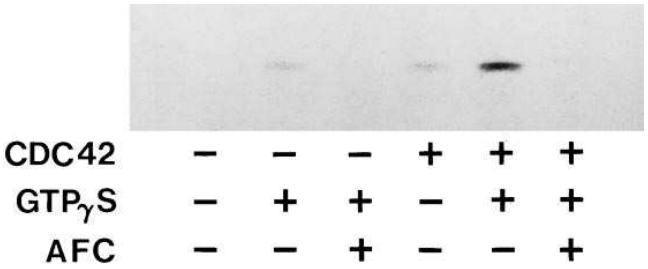

Figure 4. An autoradiogram representing the immunoprecipitation of carboxyl methylated 23-kD protein as well as of authentic CDC42. Carboxyl methylation was carried out in rat islet homogenates as described in Methods. GTPγS (10 μM), AFC (100 μM), or authentic CDC42 (1 μg) were present as indicated. After carboxyl methylation, labeled proteins were immunoprecipitated using anti-CDC42 (see Methods for additional details). Proteins were separated on SDS-PAGE (12% acrylamide) and identified by fluorography. The autoradiographic exposure time was 45 d at −70°C. After autoradiography, base-labile methanol was quantitated in the immunoprecipitates. The values of base-labile methanol released in this experiment were as follows: islet homogenate = 1.82 pmol; islet homogenate + GTPγS = 4.15 pmol; islet homogenate + GTPγS + AFC = 1.82 pmol; islet homogenate + pure CDC42 = 3.34 pmol; islet homogenate + pure CDC42 + GTPγS = 11.71 pmol; islet homogenates + pure CDC42 + GTPγS + AFC = 2.27 pmol. Data are representative of a total of four experiments (i.e., two using rat islets and two using human islets) with similar results.

ulates the carboxyl methylation of CDC42 by a second mechanism in addition to any effects on the translocation of CDC42 from cytosolic stores to membrane sites. Such effects of GTP do not seem to involve a direct stimulation of prenyl cysteine methyl transferase activity per se, since we failed to observe demonstrable effects of GTP on this enzyme in β cells (34). Rather, these findings are compatible with recent observations by several investigators (13, 44) indicating that GTP increases the affinity of G-proteins (e.g., rac2) for the prenyl cysteine methyl transferase. Limitations in the amount of pure secretory granule protein available precluded further (e.g., immunologic) characterization of this protein.

Effect of GTPγS on farnesylcysteine methyltransferase activity in insulin-secreting cells To examine the possibility that GTPγS might directly stimulate the methyltransferase activity, we quantitated the farnesylcysteine methyl transferase activity in INS-1 and HIT cells using AFC as methyl acceptor as described by us recently (34). The methyl transferase activity was predominantly associated with total particulate fraction with nearly a 16 fold higher activity in INS-1 cell particulate fraction compared with soluble fraction (i.e., 2.99±0.39 pmol/min/mg protein in the particulate fraction vs. 0.19±0.03 pmol/min/mg protein in cytosol; n = 5 determinations). Furthermore, GTPγS (100 μM) had no demonstrable direct effect on this activity in INS-1 cell homogenates (i.e., 1.26±0.05 pmol/min/mg protein in control vs. 1.41±0.12 pmols/min/mg protein in the presence of GTPγS; n = 3 determinations), compatible with recent reports of Pillinger et al. (44) and Stephenson and Clarke (45).

Immunologic identification of one endogenous carboxyl methylated 23-kD protein as CDC42. These studies demonstrated several similarities between the endogenous 23-kD protein and authentic CDC42, namely (*a*) similar mobilities on SDS-PAGE, (*b*) stimulation of carboxyl methylation by GTPγS, and (*c*) inhibition of the latter by AFC, prompting us to investigate directly, via quantitative immunoprecipitation, whether the endogenous 23-kD protein that is carboxyl methylated is CDC42. Optimization of the immunoprecipitation experimental conditions revealed that 74% of pure CDC42 carboxyl methylated in β cells was recovered in the immunoprecipitate. Under similar conditions, 71±5% of the carboxyl methylated 21–23-kD protein was immunoprecipitated using anti-CDC42 (n = 3 determinations); thus, it appears that most or all of the relevant endogenous protein reacted with anti-CDC42.

To conclusively demonstrate the specific stimulation of carboxyl methylation of endogenous 23-kD protein and of purified CDC42 by GTPγS, methylation assays were carried out using rat islet homogenates in the presence or absence of purified CDC42 under identical conditions to those described in Fig. 1 except that the labeled proteins were then immunoprecipitated using CDC42 antiserum. Fig. 4 is a representative autoradiogram (n = 2 experiments using rat islets; n = 2 experiments using human islets) of protein(s) in the immunoprecipitate after separation by SDS-PAGE, demonstrating that the endogenous 23-kD protein responds identically to authentic CDC42 (similar molecular weight; response to GTPγS; response to AFC) and is reactive with an antiserum directed against CDC42. Furthermore, base-labile methanol was quantified in the immunoprecipitates from these same preparations. GTPγS stimulated the net carboxyl methylation of endogenous 23-kD protein as well as of exogenous pure CDC42 in an AFC-sensitive manner (Fig. 4). The degree of GTPγS stimulation and AFC inhibition were comparable with that described in Fig. 1.

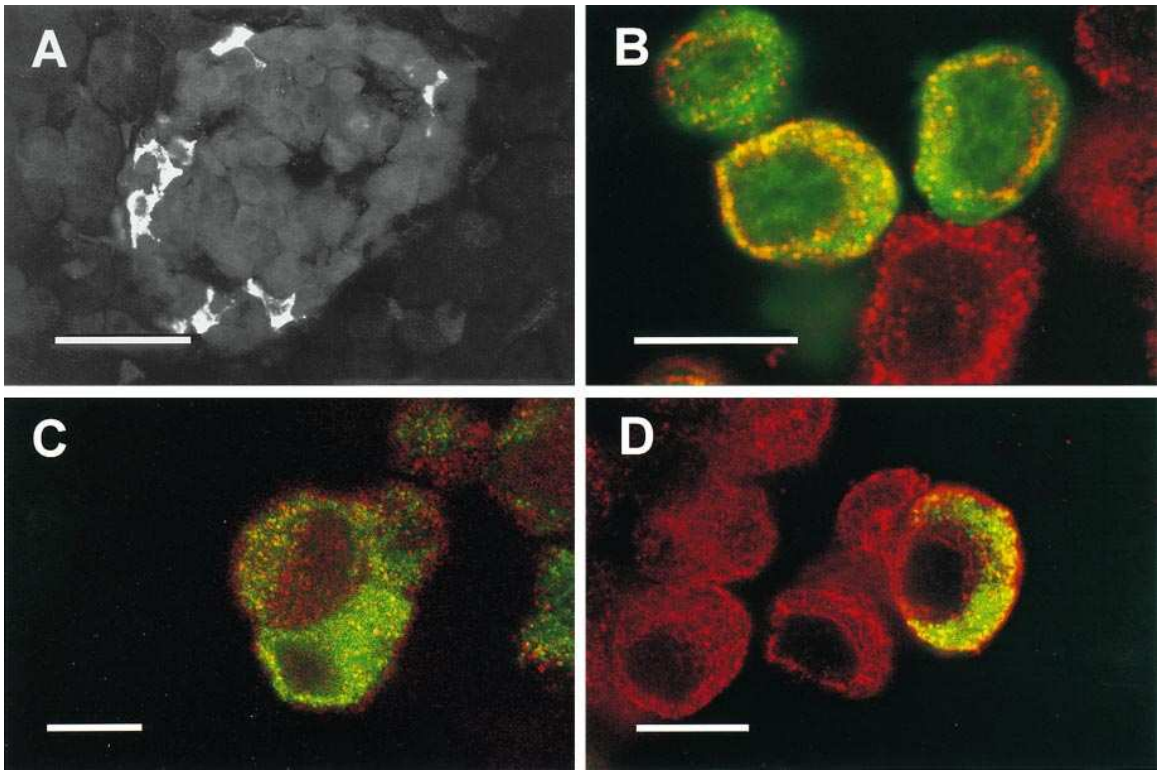

Figure 5. Localization of CDC42 in pancreatic islet cells by immunohistochemistry and by confocal microscopy. Immunohistochemical localization of CDC42 in a pancreatic tissue section (*A*) and individual cells in isolated islets (*B–D*). *A* indicates the presence of CDC42 in an islet where staining is most intense in δ cells located in the islet mantle. CDC42 was not observed in acinar cells. *B* (glucagon), *C* (insulin), and *D* (somatostatin) are merged images of single thin optical sections of CDC42 immunoreactivity (red) and the islet hormones (green). Although most granules were not associated with CDC42 (green vs. red in these images), a small subset of granules appeared to be in close association with CDC42 (yellow). Scale bar for *A*, 50 μm and for *B–D*, 10 μm.

Subcellular localization of CDC42 in islet cells by confocal microscopy. Initial experiments examined the presence of CDC42 in tissue sections from rat pancreata. Within the pancreas, CDC42 immunoreactivity was observed in the cytoplasm of all islet cells (i.e., α, β, and δ cells; Fig. 5 *A*), with the highest degree of staining in δ cells, but was not detected in acinar cells (Fig. 5 *A*). Subsequent experiments were performed using intact normal rat islets. These studies confirmed the presence of CDC42 in all islet cells (i.e., α, β, and δ cells). Staining intensity was greatest in δ cells followed by α cells and β cells. To further characterize the staining pattern for CDC42 and islet hormones within the same cells, single thin optical sections were obtained by confocal microscopy and the merged images were examined. In these experiments, the islets were stained for CDC42, insulin, glucagon, and somatostatin, examined by confocal microscopy, and the CDC42 images (red) were merged with images for the islet hormones (green) to yield a yellow color, where colocalization was demonstrable (Fig. 5, *B–D*). These studies indicated significant areas of close association and possibly some colocalization of CDC42 in the secretory granules containing specific hormones (Fig. 5, *B–D*).

GTP-dependent stimulation of the association of CDC42 with the particulate fraction and its carboxyl methylation in broken or intact cells. To determine whether GTPγS-dependent carboxyl methylation of the endogenous CDC42 results in, or is a consequence of, its association with the particulate fraction, rat islet homogenates were incubated with or without [^{3}H]SAM in the presence or absence of GTPγS and/or AFC; the carboxyl methylation of the 23-kD protein was assayed in the particulate and soluble fractions. Findings were also confirmed by an immunoblotting method. GTPγS significantly stimulated the relative proportion of methylated CDC42 localized in the particulate fraction (Table I), without any clear change in the soluble fraction. The overall methylation of CDC42 (i.e., particulate plus soluble) was stimulated by GTPγS (Table I) and this degree of stimulation was comparable with that seen in unfractionated homogenates (Fig. 1).

Additional immunoblotting studies revealed that CDC42 was predominantly cytosolic in unstimulated HIT cells (Fig. 6), compatible with recent studies by Regazzi et al. (22). Incubation of HIT cell homogenates with GTPγS resulted in an increased association of CDC42 with the particulate fraction, accompanied by a reciprocal decrease in the soluble fraction in each of the three independent experiments (e.g., Fig. 6). A similar GTPγS-dependent increase in the association of CDC42 with particulate fraction was also observed using rat islet homogenates (n = 2 independent experiments; additional data not shown), albeit without an evident stoichiometric loss of CDC42 from the cytosolic fraction. These data were confirmed by quantitating base-labile methanol in the identical experimental protein bands after immunoblotting with anti-CDC42. In these assays, GTPγS increased association of methylated CDC42 with the particulate fraction in rat islets (i.e., 0.33±0.16 pmol in the control particulate fraction vs. 1.40±

Table I. Relative Distribution of Carboxyl Methylated CDC42 in Particulate and Cytosolic Fractions of Normal Rat Islets: Effects of GTPγS

Fraction(s)	Base-labile [^{3}H]methanol released (pmol/60 min) −GTPγS	+GTPγS (10 μM)	*P*
Particulate	0.78±0.12	2.07±0.34	0.002
Cytosol	0.89±0.17	1.06±0.19	0.505
Particulate+cytosol	1.66±0.25	3.13±0.51	0.019
Particulate/particulate+cytosol	0.47±0.06	0.66±0.05	0.038

Islet homogenates were incubated with [^{3}H]SAM (100 μCi/ml) and in the absence or presence of GTPγS (10 μM) for 60 min at 37°C. After incubation, total particulate and soluble fractions were separated by centrifugation (see Methods). Proteins were separated by SDS-PAGE and degree of carboxyl methylation of the 23kD protein was quantitated by vapor-phase equilibration assay. Data are mean±SEM of 10 experiments.

0.55 pmol in the GTPγS-treated particulate fraction; $n = 6$ experiments; $P = 0.001$). However, a reciprocal reduction in the methylated CDC42 in the cytosolic fraction was again not demonstrable (i.e., 0.22±0.11 pmol in control vs 0.42±0.36 pmol in the GTPγS-treated; $n = 6$ experiments; $P = 0.225$). These data may indicate that a fraction of the methylated CDC42 may not remain firmly associated with the particulate fraction. Nonetheless, these data (Table I and Fig. 6) clearly indicate that GTPγS not only stimulates the carboxyl methylation of CDC42 but also increases its association with the particulate fraction.

The time course studies indicated that GTPγS induced a rapid stimulation (by at least as early as 5 min) of the methylated CDC42 in the particulate fractions of INS-1 cells and HIT cells. A comparable stimulation by GTPγS was demonstrable in HIT cell, INS-1 cell, and to a lesser degree in islet homogenates. The areas under time-course curves (in the absence of GTPγS) calculated by the trapezoid rule were 7,042, 6,715, and 5,385 arbitrary units (AU) for normal islets, INS-1 cells, and HIT cells, respectively; in the presence of GTPγS these values increased to 8,902, 11,742, and 9,680 AU for rat islets, INS 1 cells, and HIT cells, respectively. These data are compatible with findings described in Table I. Similar kinetics were observed in the presence of GTP (1 mM) using either normal rat islets or INS-1 cells (additional data not shown). To examine whether GTPγS mediates translocation of CDC42 to the particulate fraction independent of its effects on carboxyl methylation, the experiments described in Fig. 6 were repeated using HIT cell homogenates in the absence of SAM; AFC (100 μM) was included to inhibit endogenous methyltransferase activity. GTPγS retained its ability to stimulate (+276% of basal; $n = 2$ experiments using rat islet and 3 experiments using HIT cell homogenates) the translocation of CDC42 from the cytosolic stores to the particulate fraction even in the presence of AFC. These data are comparable with the degree of stimulation (+224% of basal) observed in the presence of GTPγS and SAM (but no AFC). These data indicate that AFC blocked the GTPγS-induced carboxyl methylation of CDC42 without blocking GTPγS-induced translocation, implying that methylation is a consequence (not a cause) of its transfer to membrane sites.

An examination of the above data indicated that even in the absence of any added GTP or GTPγS a modest association of CDC42 with the particulate fraction was demonstrable in

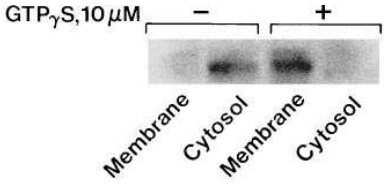

Figure 6. Immunologic identification of CDC42 as the 23-kD protein endogenous to HIT cells which is translocated to the particulate fraction under the influence of GTPγS. HIT cell homogenates (200 μg protein) were incubated with [^{3}H]SAM (100 μCi/ml) in the presence or absence of GTPγS (10 μM) at 37°C for 60 min. After incubation, total particulate and cytosolic fractions were isolated by centrifugation at 105,000 *g* for 60 min. Proteins from the total particulate and cytosolic fractions were separated by SDS-PAGE (12% acrylamide). After SDS-PAGE, proteins were transferred to a nitrocellulose membrane and protein bands corresponding to CDC42 were identified by Western blotting using a chemiluminescence kit. Results are representative of three experiments using HIT cell homogenates and two experiments using rat islet homogenates with similar results.

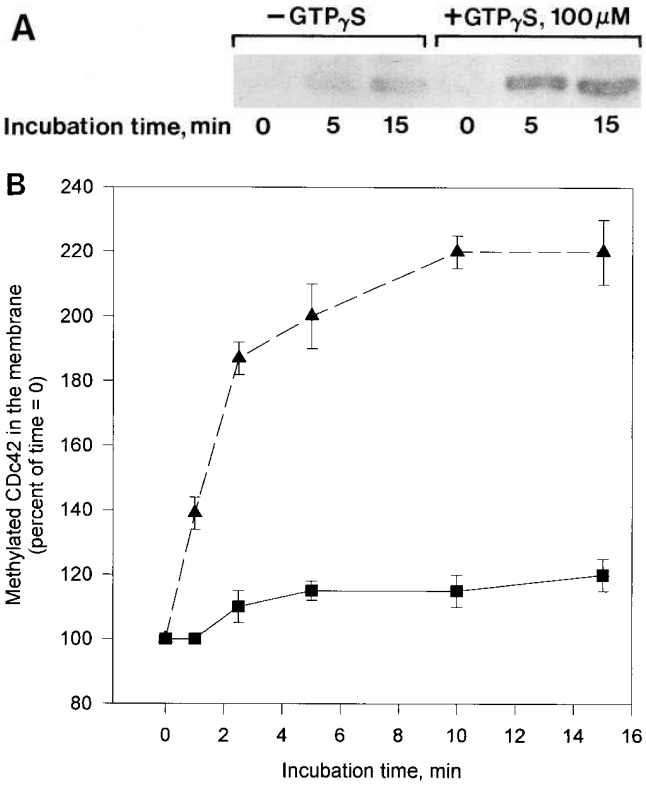

Figure 7. Time-dependent stimulation by GTPγS of translocation of CDC42 to the particulate fraction in dialyzed HIT cell homogenates. To remove endogenous GTP, HIT cell homogenates were dialyzed extensively against 20 mM Tris-HCl, pH 7.4 for 10 h at 4°C (see Methods for additional details). The ability of GTPγS (100 μM) to stimulate the translocation of CDC42 was examined by immunoblotting (*A*) and the carboxyl methylation by vapor-phase equilibration assay (*B*). Data described in *A* are representative of two experiments with similar results. Data in *B* are mean±SEM from three to four determinations. ■—■, no GTPγS; ▲— — —▲, 100 μM GTPγS.

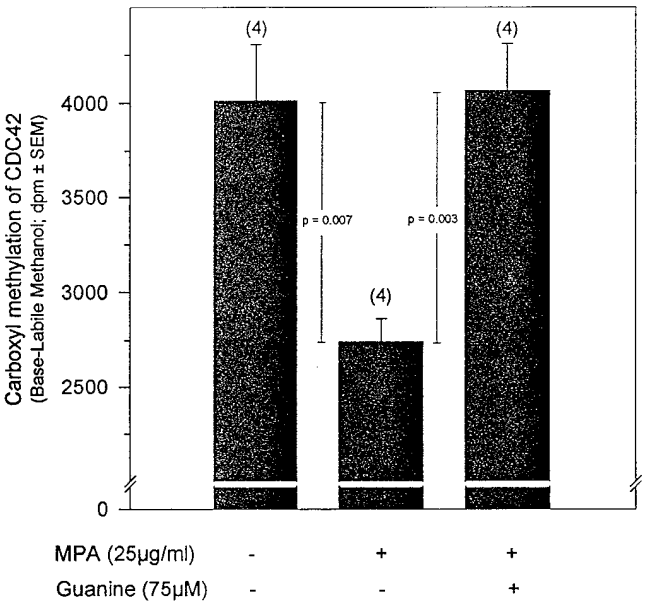

Figure 8. Depletion of intraislet GTP by MPA inhibits the carboxyl methylation of 23-kD protein in intact rat islets. Isolated rat islets (350 per group) were cultured in RPMI 1640 medium for 18 h in the presence of diluent alone, MPA alone (25 μg/ml), or MPA with guanine (75 μM). After this, islets were washed (×2) with Krebs-Ringer medium (11.1 mM glucose and 0.1% BSA) and were labeled with [^{3}H-methyl]methionine (50 μCi/ml) for 2 h at 37°C. After this incubation, islets were homogenized, proteins were separated by SDS-PAGE (12% acrylamide) and the carboxyl methylation of 23-kD protein was quantitated by vapor-phase equilibration assay. These data indicated significant inhibition of the carboxyl methylation of 23-kD protein in islets treated with MPA and a complete reversal of such an inhibition by coprovision of guanine. Data are mean±SEM of four independent experiments.

normal rat islets, HIT cells, or INS-1 cells. Since this may be, in part, due to the endogenous GTP in these preparations, endogenous GTP was depleted by extensive dialysis of cell-free preparations (see Methods). Specific association of CDC42 with the particulate fraction was then quantitated by immunoblotting (Fig. 7 *A*) or by base-labile methanol assay (Fig. 7 *B*). A minimal degree of translocation of CDC42 occurred in the absence of GTPγS even in dialyzed HIT cell homogenates. When GTPγS was added to the dialyzed preparations, a significant (1.9-fold) increase in the membrane association of CDC42 was demonstrable within 5 min of exposure (Fig. 7 *B*).

Leiser et al. (46) very recently reported GTP-dependent carboxyl methylation in βTC3 cells of a 23-kD protein which exhibited certain similarities (i.e., molecular size and isoelectric point) with rap 1, another low molecular weight G-protein. However, these investigators did not immunoprecipitate the carboxyl methylated 23-kD protein using antisera directed against rap 1. Using intact rat islets as well as HIT cells, we have confirmed these observations that rap 1 is also carboxyl methylated in a GTP-sensitive manner. However, using immunoblotting, we observed that rap 1 was predominantly associated with the particulate fraction; exposure to GTPγS (in the presence of AFC; 100 μM) did not result in any significant alterations in the distribution (i.e., particulate versus cytosolic) of this protein at early (60 s) or late (45 min) time points (additional data not shown). These data may suggest different regulatory roles for rap 1 and CDC42 in insulin secretion (see below).

These data regarding the stimulatory effects of exogenous GTP on the translocation and the carboxyl methylation of CDC42 prompted us to examine the role of endogenous GTP in the regulation of such events in intact islets. For this purpose, intraislet GTP was depleted by culturing islets in the presence of MPA (2, 3) followed by quantitating the carboxyl methylation of CDC42 in these islets after pre-labeling with [^{3}H]methionine. MPA pretreatment significantly reduced (−32%; $n = 4$ experiments; $P = 0.007$) the carboxyl methylation of CDC42 in islet homogenates (Fig. 8). This reduction of carboxyl methylation of CDC42 was observed in the particulate fraction alone, as well as in total homogenates (data not shown). Furthermore, selective repletion of GTP in MPA-treated islets by coprovision of guanine (2, 3) completely reversed the inhibition of the carboxyl methylation of CDC42 seen after GTP depletion to values indistinguishable from control islets (Fig. 8). Mycophenolic acid had no demonstrable effects on the uptake of [^{3}H]methionine (additional data not shown). In contrast, GTP depletion did not decrease (+3% of control; $n = 3$) the carboxyl methylation of the catalytic subunit of protein phosphatase 2A (47). However, MPA treatment did not detectably alter the subcellular distribution of CDC42 in soluble versus particulate fractions, as assessed by immunoblotting (additional data not shown).

Effects of agonists of insulin secretion on the carboxyl methylation of specific 23-kD proteins in HIT cells: effects of GTP depletion. For this purpose, intact HIT cells were prelabeled with [^{3}H]methionine and exposed to either high (11.1 mM) glucose or depolarizing concentrations (40 mM) of KCl for different time intervals. Either glucose (Fig. 9 *A*) or potassium (Fig. 9 *B*) stimulated (2.5–3-fold) the total carboxyl methylation of the 23-kD protein band. These effects were demonstrable within 15-30 s of exposure to these agonists. Similar stimulatory effects were also demonstrable in intact rat islets (data not shown), an effect no longer demonstrable in the presence of mannoheptulose (30 mM), a metabolic blocker of glucose metabolism (112% of control in normal rat islets; three determinations). Furthermore 3-*O*-methyl glucose, a transportable but nonmetabolizable analogue of glucose, failed to stimulate the carboxyl methylation of the 23-kD protein (+7% of control in rat islets [$n = 3$ determinations] or HIT cells [$n = 4$ determinations]).

Additional studies were then carried out to examine effects of these agonists on the specific carboxyl methylation of CDC42 and rap 1. For this purpose, experiments were repeated under conditions similar to those described in Fig. 9 except that CDC42 or rap 1 were immunoprecipitated from these cells. Data in Fig. 10 (*A*) indicate that high glucose (11.1 mM) significantly ($P = < 0.001$ vs. basal; df = 19) stimulated the carboxyl methylation of CDC42; however, there was no demonstrable effect of a depolarizing concentration of potassium on the carboxyl methylation of CDC42 in intact HIT cells ($P = 0.284$ vs. basal; df = 18). Interestingly, however, as shown in Fig. 10 (*B*) , either glucose or potassium stimulated the carboxyl methylation of rap 1 ($P = 0.007$ vs. basal; df = 6 and 0.014 vs. basal; df = 8 for glucose and potassium, respectively). Based on these data it seems likely that rap 1 carboxyl methylation may be required for a calcium-dependent event(s), a conclusion compatible with observations of Leiser et al. (46) indicating inhibition by D-600 (a calcium channel blocker) of

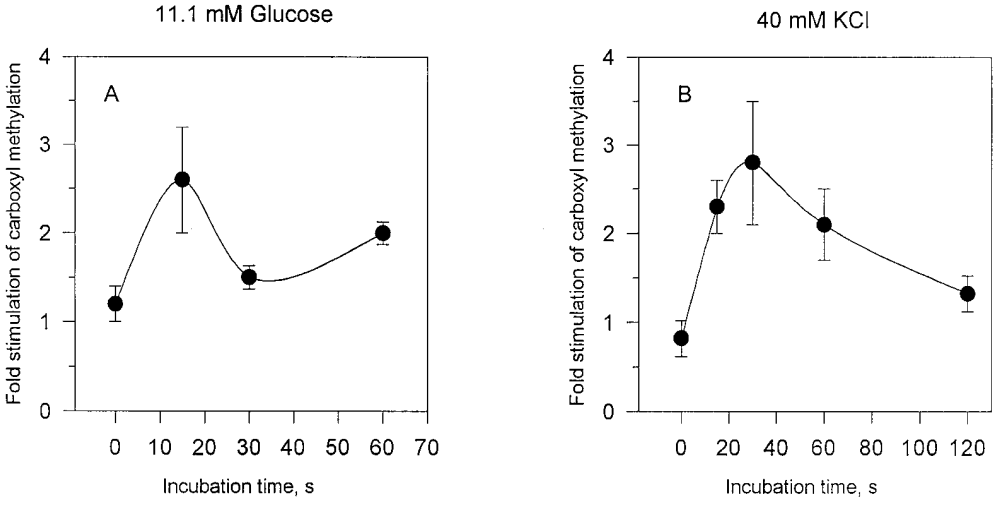

Figure 9. Time-dependent stimulation by glucose and potassium of the carboxyl methylation of 23-kD proteins in intact HIT cells. Intact HIT cells were prelabeled with [^{3}H]methionine for 1 h at 37°C in the presence of 0.1 mM glucose as described in the text. After this, cells were washed quickly with an isotonic medium and were incubated with either glucose (11.1 mM; *A*) or a depolarizing concentration of KCl (40 mM; *B*) for different time intervals as indicated. Reaction was terminated by addition of TCA (10% final concentration) and proteins were separated by SDS-PAGE. The degree of carboxyl methylation of 23-kD protein was assessed by vapor-phase equilibration assay. Data were expressed as fold stimulation in the carboxyl methylation of 23 kD by glucose or potassium over the basal methylation of 23-kD protein observed in the presence of 0.1 mM glucose alone at the same points. Data are mean±SEM from three determinations at each time point.

glucose- and potassium-induced stimulation of the carboxyl methylation of a 23-kD protein (which comigrated with rap 1) in βTC3 cells.

Since we have shown (2, 3) that depletion of intraislet GTP (by MPA) results in inhibition of nutrient-induced, but not K^+-induced, insulin secretion, we examined, by immunoprecipitation using specific antisera, the effects of glucose on the carboxyl methylation of CDC42 in HIT cells in which GTP had been depleted. Glucose-induced stimulation of the carboxyl methylation of CDC42 (Fig. 11, *left two columns*) was no longer demonstrable in GTP-depleted HIT cells (Fig. 11, *middle two columns*). However, coprovision of guanosine (500 μM) with MPA completely restored the stimulatory effects of glucose on the carboxyl methylation of CDC42 (Fig. 11, *right two columns*). These data are compatible with data obtained using rat islets (Fig. 8).

Effects of protein methylation inhibitors on nutrient-induced insulin secretion. Under static incubation (for 45 min) conditions, AFC (100 μM), but not AGC (100 μM), inhibited glucose (16.7 mM)-induced insulin secretion from normal rat islets by 46±12% (n = 6 experiments; $P < 0.001$), confirming our earlier observations (19). Additionally, in perifused islets, AFC (100 μM) significantly inhibited glucose-induced insulin secretion (Fig. 12). In the initial perifusion studies, there was no apparent effect of AFC on the first phase (1–10 min) of glucose-induced insulin secretion; however, AFC markedly reduced the second phase of glucose-induced insulin secretion, in each of four experiments. It is possible that the apparent

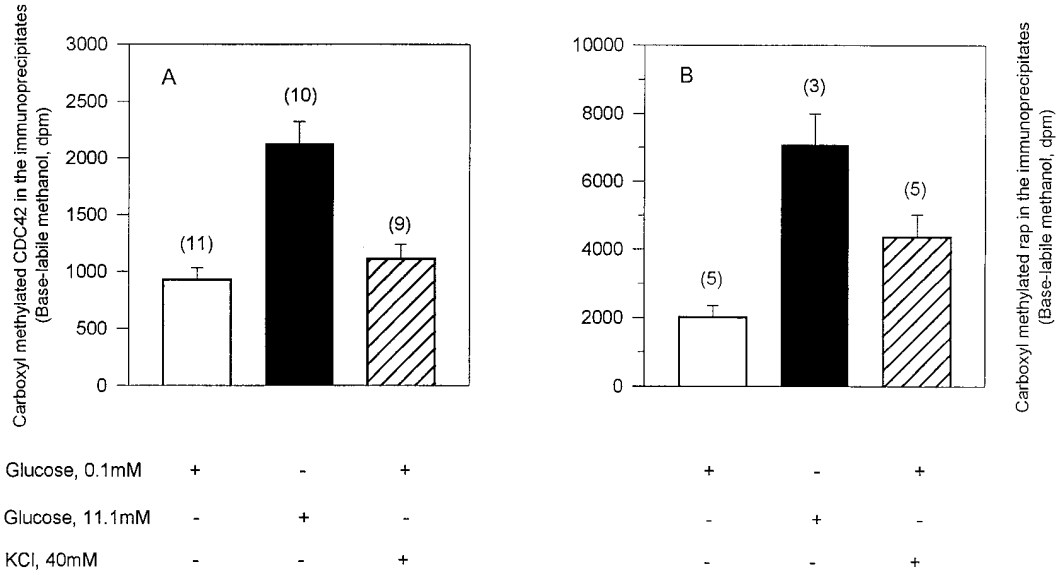

Figure 10. Immunoprecipitation of CDC42 and rap 1 from intact HIT cells stimulated with glucose or potassium. HIT cells were prelabeled with [^{3}H]methionine for 1 h at 37°C as indicated in the text and then exposed to glucose (11.1 mM) for 15 s or KCl (40 mM) for 30 s. CDC42 (*A*) or rap 1 (*B*) were immunoprecipitated (see text for details), and their carboxyl methylation was assessed by base-labile methanol release assay. Data are mean±SEM for the number of determinations indicated in the figure. Glucose stimulated (*A*) the carboxyl methylation of CDC42 (P = 0.0001 vs. control; df = 19). In contrast, KCl failed to exert any significant effects on the carboxyl methylation of CDC42 (P = 0.284; df = 18). Both glucose and KCl augmented the carboxyl methylation of rap 1 (P = 0.007 [df = 6] and 0. 0136 [df = 8] vs. control, respectively).

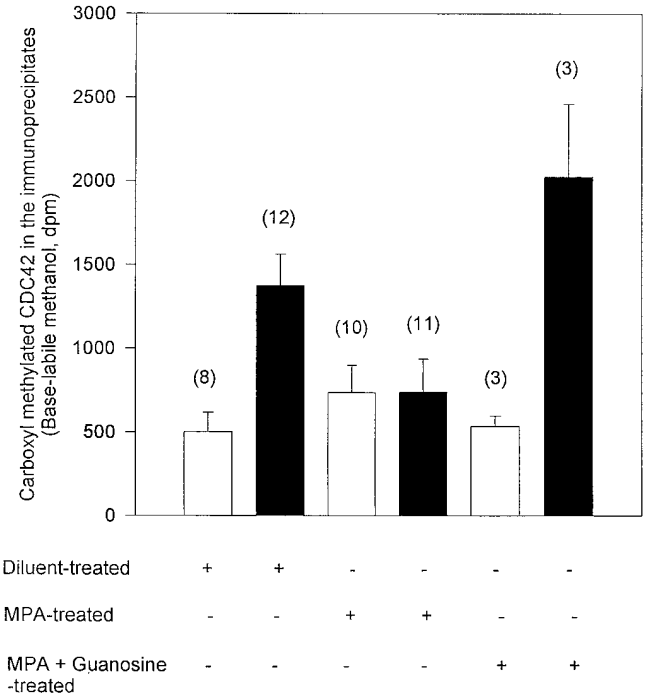

Figure 11. Inability of glucose to stimulate the carboxyl methylation of CDC42 in GTP-depleted HIT cells: its reversibility upon GTP repletion. HIT cells (20×10^6 per group) were incubated with either diluent alone, MPA alone (1 μg/ml), or MPA plus guanosine (500 μM) for 6 h at 37°C. After this, cells were prelabeled with [^{3}H]methionine, exposed to 0.1 mM glucose (*open bars*) or 11.1 mM glucose (*closed bars*) for 15 s, and CDC42 was immunoprecipitated as described in Methods. Agarose-associated proteins were separated by SDS-PAGE and the degree of carboxyl methylation of CDC42 was quantitated by vapor-phase equilibration assay. These data indicated that glucose stimulated the carboxyl methylation of CDC42 only in control cells, but not GTP-depleted (i.e., MPA- treated) cells. However, repletion of GTP by coprovision of guanosine completely restored glucose's stimulatory effects, implying a requirement for endogenous GTP in glucose-induced carboxyl methylation of CDC42 in insulin-secreting cells. $P = 0.02$ (df = 21) for control vs. MPA-treated cells; 0.005 (df=12) for MPA vs. MPA plus guanosine; and 0.155 (df =13) for control vs. MPA plus guanosine.

lack of inhibition of the first phase (up to 10 min) of glucose-induced insulin secretion in the perifusion experiments could represent a delay in the transport of AFC into intact cells (19, 30). Therefore, islets were preincubated with AFC (100 μM) for 60–75 min (to increase transport of AFC), and then its effects on glucose (16.7 mM)- or ketoisocaproate (20 mM)-induced insulin secretion were restudied during incubations of 5 or 10 min, which would be comprised largely of first phase secretion. AFC blocked glucose- and ketoisocaproate-induced first phase secretion by 41±8% and 40±8%, respectively (n = 5 experiments in each case; $P = 0.02$). The degree of inhibition was comparable with AFC inhibition of glucose-induced insulin secretion during 45-min incubations (19). However, AFC (100 μM) failed to inhibit K^+ (40 mM)-induced insulin secretion either during a 10-min incubation (+2%; this study) or during 45-min incubations (19), suggesting that AFC probably does not block calcium-dependent exocytosis directly (see below). AFC (100 μM) also inhibited both glucose- or glucose plus forskolin–induced insulin secretion from pure β (HIT) cells (−63 to 71%; n = 6 determinations) without significantly affecting (+3%; n = 6 determinations) K^+-induced secretion. There was no significant effect of AFC on islet insulin content during 10-min (27±1 mU/ml in control vs. 23±1 mU in AFC-treated islets; df = 33) or 45-min incubations (19±2 mU/ml in control vs. 25±4 mU in AFC-treated islets; df = 4).

Effects of carboxyl methylation inhibitors on glucose-activated phosphoinositide turnover in normal rat islets. Metabolizable nutrients, such as glucose, mannose, or glyceraldehyde increase phosphoinositide turnover and the production of inositol phosphates in isolated islets in a fashion felt to be critical to the induction of insulin secretion (38, 48, 49). Studies by Vallar et al. (50) indicated that provision of GTPγS to permeabilized clonal β cells provoked calcium-independent, GDPβS-inhibitable phospholipase C activation, and insulin secretion. Additionally, it has been shown recently (39) that depletion of intracellular GTP in islets by using MPA resulted in a significant reduction (−63%) in glucose-activated phosphoinositide turnover. Therefore, we studied the effect of the blockade of G-protein carboxyl methylation on glucose-induced phosphoinositide turnover, as assessed by a validated perifusion technique (39). Glucose (16.7 mM) stimulated the fractional efflux of [^{3}H]inositol from normal rat islets in a time-dependent manner (Fig. 13); inclusion of AFC (100 μM) markedly reduced (−49%) this effect (Fig. 13). AGC (100 μM), an inactive analogue of AFC, affected glucose-induced inositol efflux only insignificantly. The areas under the curves representing [^{3}H] inositol efflux were 28±5 AU (n = 4), 14±3 AU (n =4), and 29±2 AU (n = 3) for glucose (16.7 mM), glucose plus AFC (100 μM), and glucose plus AGC (100 μM), respectively. We have observed previously that exposure of normal rat islets to high glucose (16.7 mM) leads to hydrolysis of PIP_2, PIP, and PI (39). In the current studies, we observed that pretreatment of islets using AFC (100 μM) blunted this response to glucose, as assessed by an increase (+18%) in PIP_2 content (3,074±81 dpm in the absence of AFC vs. 3,611±146 dpm in its presence; $P < 0.02$; n = 3 experiments). Generally similar inhibitory effects of AFC on the hydrolysis of PIP (+23% increase in content) and PI (+22% increase in content; n = 3 experiments) were demonstrable under these conditions. In contrast, AGC failed to preserve the content of phosphoinositides in glucose-stimulated islets.

Discussion

The principal novel findings of the current study are: (*a*) that glucose, a nonreceptor agonist, promotes the carboxyl methylation of CDC42 in intact β cells; (*b*) that ambient GTP concentrations can be regulatory for the activation of at least one G-protein (CDC42) in both broken and intact cells; and (*c*) that the carboxyl methylation of CDC42 (and possibly other G-proteins) may facilitate its interaction with one or more effector molecules (such as phospholipase C or its substrates) thereby facilitating their activation. This effect likely explains at least a part of the permissive role of GTP in relatively proximal steps in the cascade of nutrient-induced insulin release (2, 3).

Many low molecular mass G-proteins (including the γ subunits of heterotrimeric G-proteins) possess unique carboxyl terminal amino acid sequences necessary for a series of post-translational modification reactions, such as isoprenylation, carboxyl methylation, and palmitoylation (8–10). Recent immunologic studies (5, 7, 22, 51) have identified some of the G-proteins in normal rat islets, human islets, and insulin-

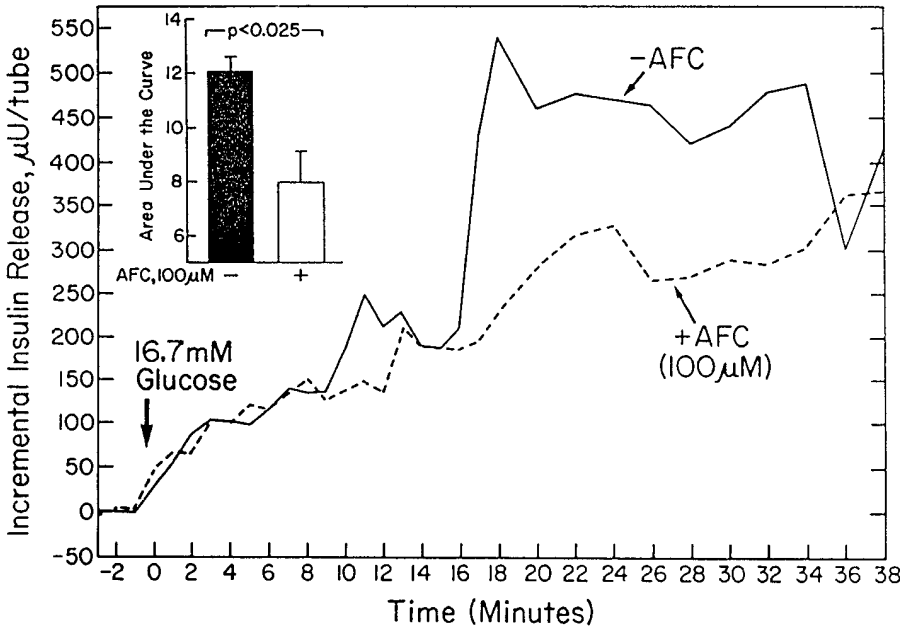

Figure 12. Inhibition by AFC of glucose-induced insulin secretion from normal rat pancreatic islets under perifusion conditions. Perifusions were carried out in the presence of 16.7 mM glucose and in the absence or presence of 100 μM AFC (see Methods). Data are representative of four experiments with similar results. Inset depicts the mean±SEM of the areas (arbitrary units) under the curves for insulin secretion in the absence (*closed bar*) or presence (*open bar*) of AFC. AFC inhibited glucose-induced insulin secretion in each of the four experiments ($P < 0.025$).

secreting transformed pure β (HIT or RIN) cells as species of rab, CDC42, rac2, rho, rap, and ADP-ribosylation factor (ARF). Using [^{14}C]mevalonic acid as a precursor for isoprenoid groups, we demonstrated the isoprenylation of at least five low molecular weight G-proteins (19) in normal rat islets; these were enriched in the secretory granule fraction (4, 5, 7). Moreover, recent studies demonstrated the carboxyl methylation of at least four proteins (~ 36–38 kD, 23 kD, 21 kD, and < 8 kD) in normal rat islets and pure β (HIT or INS-1) cells. Of these, the carboxyl methylation of only two proteins (i.e., 21- and 23-kD proteins) was stimulated by GTP (19). However, the GTPγS-stimulated carboxyl methylation of the 21-kD protein was resistant to inhibitory effects of AFC (19).

In each of five different classes of insulin-secreting cells this protein was carboxyl methylated. Addition of purified CDC42 significantly increased the carboxyl methylation of this protein. The carboxyl methylation of the endogenous 23-kD protein as well as of exogenous purified CDC42 was stimulated in a guanine nucleotide-specific manner in all five insulin-secreting cell types studied (albeit to varying degrees). The differences in the degree of carboxyl methylation of CDC42 between the different preparations may, in part, be due to variations in their endogenous GTP and/or CDC42 content. Indeed, recent studies have indicated significant differences in the abundance and distribution of G-proteins, including CDC42, between normal rat or human islets, and insulin-secreting β (HIT or RIN) cells (5, 7). Similar degrees of stimulation of the carboxyl methylation of the 23-kD protein and of CDC42 were demonstrable by immunoprecipitation experiments (Fig. 4). These data are compatible with recent observations by Backlund (14, 52) regarding the stimulation of the carboxyl methylation of CDC42 (G25K) by GTPγS.

Since AFC inhibited nutrient-induced (but not K^+-induced) insulin secretion from isolated islets and pure β cells under static and perifusion conditions, and since GTP promotes nutrient-induced (but not K^+-induced) insulin secretion (2), these data suggest a key regulatory role for GTP-dependent carboxyl methylation of CDC42 in nutrient-induced (but not calcium-induced) insulin secretion. Possible roles for the carboxyl methylation of rap 1 (46) and γ subunit (reference 43 and Kowluru, A., and S.A. Metz, manuscript in preparation) in insulin release are also likely, as suggested by Leiser et al. (46) but probably at a different site (or sites) in exocytosis. Thus, while either glucose or K^+ increased the carboxyl methylation of rap 1, only glucose increased the carboxyl methylation of CDC42. Leiser et al. (46) have also observed that both glucose and K^+ increased the carboxyl methylation of a 23-kD protein

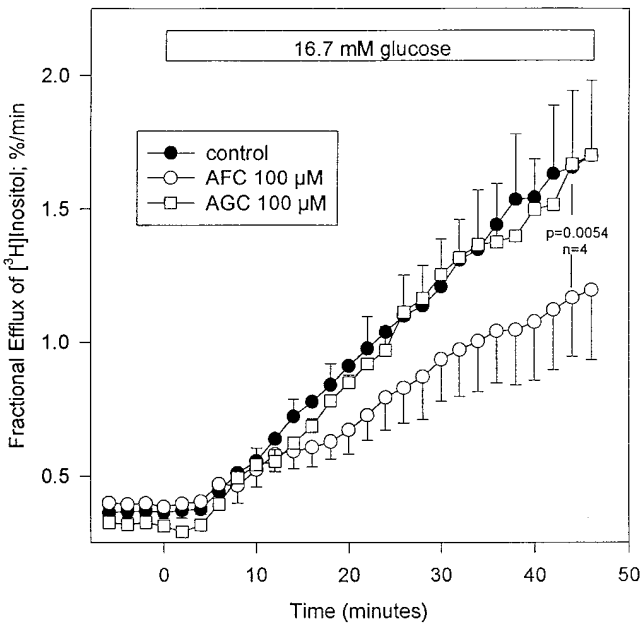

Figure 13. Inhibition by AFC of glucose-induced inositol efflux from normal rat islets under perifusion conditions. Perifusions were carried out in the presence of 16.7 mM glucose alone or in the presence of 16.7 mM glucose and either 100 μM AFC or 100 μM AGC (see Methods for additional details). Data are mean±SEM of five experiments in the presence of glucose alone, four experiments with AFC, and three experiments using AGC. *P* for control vs. AFC-treated = 0.0054 and control vs. AGC-treated = 0.852.

but these investigators did not identify the protein immunologically. However, these investigators demonstrated the carboxyl methylation of rap 1. The current studies suggest the formulation that glucose increases the carboxyl methylation of CDC42 which, in turn, stimulates phospholipase C activity resulting in insulin secretion; in contrast, rap 1 carboxyl methylation may be required for an unidentified, calcium-induced event.

Using detergent phase–partitioning technique, Regazzi et al. (22) demonstrated that CDC42 is hydrophobic in HIT cells, which identifies it as a prime candidate for isoprenylation and carboxyl methylation reactions. Moreover, in the same study, they demonstrated that inhibition of the isoprenylation of G-proteins (by lovastatin) resulted in a selective accumulation of CDC42 in cytosol, in contrast to other low molecular mass G-proteins (e.g., rho and ARF). These data are in accord with our recent observations suggesting that treatment of intact islets with lovastatin resulted in the cytosolic accumulation of low molecular weight G-proteins with a concomitant reduction in nutrient-induced insulin secretion (19). Generally similar conclusions were reached by Li et al. (53) using HIT cells. Taken together, these findings suggest that prenylation augments the net retention of CDC42 in the particulate fraction; carboxyl methylation further increases its hydrophobicity (8–10) and might strengthen its affinity for membranes and/or effector proteins. The association of CDC42 (but perhaps not rap 1) with the membranes and/or with their intrinsic effectors may be an obligatory step for nutrient-induced insulin secretion, since inhibition of isoprenylation (by lovastatin) or of carboxyl methylation (by AFC) was followed by a marked reduction in nutrient-induced insulin secretion.

While there are extant data demonstrating the isoprenylation (54) and carboxyl methylation of G25K or CDC42 (14, 52), the data presented herein clearly demonstrate that GTPγS directly stimulates association of CDC42 with the particulate fraction in five insulin-secreting cells. This conclusion is based on two independent methods: first, evidence of translocation from soluble to particulate fractions, as assessed by immunoblotting; and second, an increase in the percentage of methylated CDC42 associated with the particulate fraction, as determined using vapor-phase equilibration assay. These data are in agreement with several recent studies (15–18) demonstrating the GTPγS-mediated translocation of other small molecular weight G-proteins such as rac2 and ARF. Furthermore, as has been observed by Bokoch et al. (15), GTPγS-induced translocation of CDC42 does not appear to require carboxyl methylation, since we observed stimulation by GTPγS of the membrane association of CDC42 even in the presence of AFC. Based on these data, it seems likely that the carboxyl methylation of CDC42 is, to a large degree, a consequence, rather than a cause, of its membrane association.

However, it is likely that GTPγS has additional stimulatory effects on the carboxyl methylation of CDC42 in addition to its translocation. To address this issue, we first studied the methyl transferase itself. Prenyl cysteine methyl transferase activity was absent from the soluble fraction, compatible with its membranous distribution in other cells (13, 14, 30, 34, 44, 45). GTPγS did not appear to directly stimulate methyl transferase activity using AFC as a methyl acceptor. These data are also compatible with recent reports of the lack of demonstrable effects of GTP on methyltransferase activity in other cell types (44, 45). However, we cannot rule out the possibility that GTPγS might stimulate the enzyme when a protein-bound prenyl cysteine is used as substrate (in contrast to an artificial substrate such as AFC). However, there must be still other loci in the CDC42 methylation cascade at which GTPγS could exert its stimulatory effects, since GTP stimulated the carboxyl methylation of CDC42 in the isolated secretory granule fraction. One possibility is that binding of GTPγS to CDC42 augments its affinity for the methyltransferase, as has been reported for rac2 (13). A second possibility is that GTPγS promotes the dissociation of CDC42 from its regulatory binding proteins such as GDI (GDP-dissociation inhibitor), thereby opening its carboxyl terminus to the methyl transferase (55). Although recent studies by Regazzi et al. (22) argue against a direct effect of GTPγS acting alone, other studies have suggested that GTPγS acts in concert with an unidentified factor to release GDI (15). Recent studies of rab 5 (56) and rab 9 (57) have suggested that three distinct steps in the translocation of G-proteins can be identified: first, dissociation of GDI, which may "chaperone" G-proteins to relevant membranes; second, loose association with the membrane; and third, binding of GTPγS (presumably followed by methylation and tight membrane association). Further studies will be needed to fully elucidate the correct sequence in β cells.

Data from our studies indicate significant differences in the time-dependent stimulation of the carboxyl methylation of CDC42 in intact cells by glucose (demonstrable within few seconds) and in broken cell preparations by GTPγS (observed within minutes). It is possible that in an intact β cell, the transient nature of the stimulatory effects of glucose (and potassium) might represent rapid turnover of cysteic acid methyl esters on CDC42 (and rap 1) involving a rapid stimulation of methylation and demethylation of these proteins. Indeed, in support of such a formulation we obtained evidence to indicate localization of cysteinyl (43) and leucyl (47) methyl ester hydrolytic activities in pancreatic β cells. In addition to these, glucose might also rapidly modulate the activity of other G-protein regulatory proteins such as GDI or GTP/GDP exchange proteins leading to rapid effects not reproduced by GTP alone. Certain regulatory factors may be perturbed/lost in broken cells during homogenization, resulting in a delayed demethylation of CDC42.

A 23-kD protein was methylated in secretory granule fractions of rat islets and insulinoma cells in a GTPγS- and AFC-dependent manner. Furthermore, the degree of stimulation by GTPγS of carboxyl methylation of this protein was greater in the secretory granule fraction six to sevenfold compared with what was observed in homogenates (up to 3.5-fold). Recent studies by Ziman et al. (58) in *Saccharomyces cerevisiae* suggest a critical role for CDC42 in secretory vesicle fusion with the plasma membrane. Using immunofluorescence techniques, these investigators localized CDC42 to the plasma membrane in close proximity to secretory vesicles at the site(s) of bud emergence. By analogy, localization of this protein on secretory granules of β cells (as suggested by the carboxyl methylation data) might place it in a strategic position to mediate a critical step in insulin secretion. In addition, the localization of CDC42 by confocal microscopy not only to β cells but also to α- or δ cells (Fig. 5), and even possibly on their secretory granules, suggests that possible effects of CDC42 on paracrine interrelationships in islets (i.e., the secretion of glucagon or somatostatin) merit further investigation to fully explain its role in insulin release.

Data from insulin release experiments indicate a marked inhibition by AFC of glucose-induced insulin secretion from

normal rat islets under static and perifusion conditions; these findings were confirmed using another inhibitor of carboxyl methylation (e.g., homocysteine and 3-deaza-adenosine). AFC also inhibited ketoisocaproate-induced insulin secretion, thus excluding glucose metabolism as the locus of AFC inhibition. However, in contrast to nutrients, the effects of agonists which induce secretion directly by activating distal components in signal transduction (such as a phorbol ester or mastoparan) are not inhibited by AFC (19); since the stimulatory effects of 40 mM K^+ were also resistant to inhibition by AFC (19), it appears that calcium-dependent fusion and exocytosis may not require the carboxyl methylation of G-proteins. Although CDC42 does not appear to be involved in calcium-dependent exocytotic events (e.g., fusion or fission), its possible presence on secretory granules might indicate a role for this protein in secretory granule biogenesis or margination.

It may be noted in passing that recent studies have cast some doubt on the specificity of AFC as a functional probe of carboxyl methylation (59–61). However, in those studies, nonspecific effects of AFC were seen on receptor-activated events and were linked to actions on heterotrimeric G-proteins; in our studies, AFC inhibited the carboxyl methylation of monomeric G-proteins and blocked nutrient-activated (i.e., receptor-independent) events, while lacking effects on receptor-dependent modifiers of insulin secretion (Metz, S.A., and M.E. Rabaglia, unpublished data). Furthermore, AFC failed to inhibit insulin secretion induced by 40 mM K^+, a protein kinase C activator, mastoparan (19), or carbachol (Metz, S.A., unpublished observations), arguing against nonspecific inhibitory effects of AFC on exocytosis. Additionally, the effects of AFC were mimicked by 3-deazaadenosine and *S*-adenosyl homocysteine (which are structurally distinct from AFC; see Results), but not by a congener of AFC (i.e., AGC) which did not block prenyl cysteine methylation in insulin-secreting cells (19). Similar effects were also seen with lovastatin (19) which should reduce the carboxyl methylation of G-proteins by inhibiting the requisite antecedent prenylation step. Together, these observations strongly indicate a selective blockade of a nutrient-activated step(s) in stimulus–secretion coupling by carboxyl methylation, distal to fuel metabolism, but relatively proximal in the stimulus–secretion cascade. Data obtained using α-ketoisocaproate exclude the early steps in glucose metabolism as this site (although we cannot categorically exclude steps in fuel metabolism at the mitochondrial level). One key step critical to nutrient-induced (but not K^+- or phorbol ester–induced) insulin secretion is activation of phospholipase C. We speculated that carboxyl methylation either stabilizes the translocation/membrane insertion of CDC42 or related G-proteins (by increasing their lipophilicity and/or neutralizing a negative charge of the carboxylate anion on their carboxyl termini) or directly increases their interaction with their putative membrane receptor, effector protein(s), or activating proteins such as GDP/GTP-exchange proteins (62). Indeed, AFC (but not AGC) reduced glucose-induced phosphoinositide hydrolysis. The findings that one or more low molecular mass G-proteins may facilitate phospholipase C activation are reminiscent of recent findings by Bowman et al. (63) suggesting that an unidentified member(s) of the rho family, perhaps including CDC42, may activate phospholipase D in neutrophils. These conclusions that carboxyl methylation increases association of small G-proteins with one (or more) effectors or activators may be analogous to the requirement of a-factor mating pheromone for carboxyl methylation to exert bioactivity (64). Furthermore, other hydrophobic modifications such as palmitoylation (65) or prenylation (66) have been shown to increase the interaction of G-proteins with phospholipase C. Other very recent studies (67, 68) provide precedent for the formulation that alterations in the hydrophobicity of GTP-binding proteins, achieved via inhibition of their posttranslational modifications (e.g., prenylation or palmitoylation), can interfere with their coupling to effector systems including phospholipases. Since AFC inhibited glucose-induced phospholipase activity and insulin release, but not the membrane association of CDC42 or rap 1, it appears that the latter may be required, but not sufficient, for the activation of relevant effectors. Although one relevant effector for one (or more) small G-proteins in the β cell appears to be phospholipase C, other additional possibilities are not excluded. These data also do not, of course, exclude a role(s) for other G-proteins (e.g., rho, rac2, rab3A) present in the β cells in the stimulus–secretion coupling of agonist-induced insulin secretion. Together, such roles for G-proteins could explain much of the permissive requirement for GTP in physiologic insulin secretion (2, 3).

If the formulation is correct that the GTP-dependent carboxyl methylation of G-proteins such as CDC42 is required for maximal activation of fuel-induced phosphoinositide hydrolysis, and the consequent insulin release, one might anticipate that a marked reduction in endogenous GTP content of islets (achieved through the use of the GTP synthesis inhibitor, MPA) would impede these same processes. Indeed, these postulates were fulfilled, since MPA: (*a*) markedly reduced GTP content and GTP/GDP ratios of islets (2, 3) and pure β cells (Meredith, M., G. Li, and S.A. Metz, unpublished observations); (*b*) reduced the carboxyl methylation of endogenous CDC42 (present study); (*c*) impaired phospholipase activation (39), and (*d*) selectively impeded glucose- or ketoisocaproate–induced (but not K^+-induced) insulin release in isolated islets (2, 3). If this postulated cascade of events is correct, it would, in fact, indicate that the activity of at least some G-proteins can be significantly modulated at the level of ambient GTP concentration.

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