Characterization of prenylcysteine methyltransferase in insulin-secreting cells

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Prenylcysteine carboxymethyltransferase, an enzyme involved in the post-translational modification of many signalling proteins, was characterized in insulin-secreting INS-1 cells and normal rat pancreatic islets. The activity of this enzyme was monitored by the methylation of an artificial substrate (a prenylated cysteine analogue) with *S*-adenosyl[methyl-³H]methionine as methyl donor. More than 95% of the methyltransferase activity was associated with the membranes, and high-salt treatment only partially extracted the enzyme from the membranes. The highest specific activity was in the insulin-granule-enriched 25000 g pellet obtained by differential centrifugation. However, a highly purified insulin-enriched fraction obtained by density centrifugation in Percoll did not exhibit methyltransferase activity. The analyses of marker enzymes for cellular organelles revealed that the methyltransferase was co-localized with the plasma membrane and probably the endoplasmic reticulum, but not

with the mitochondria or lysosomes. Guanosine 5'-[γ-thio]triphosphate failed to increase methyltransferase activity directly, although it promotes the methylation of GTP-binding proteins. Mastoparan, Ca²⁺, cAMP and the protein kinase C activator phorbol 12-myristate 13-acetate did not alter enzyme activity. In addition, methyltransferase activity was not stably modified by stimulation of intact cells using glucose or other agents. However, the carboxymethylation of certain low-molecular-mass G-proteins is increased by glucose stimulation; conversely, treatment of cells with *N*-acetyl-*S*-trans,trans-farnesyl-L-cysteine inhibited glucose- and forskolin-induced insulin secretion. These results suggest that the membrane-associated prenylcysteine carboxymethyltransferase may be constitutively active and that the methylation of target proteins *in vivo* is regulated by the access of these proteins to the methyltransferase, as well as by their active (GTP-liganded) configuration.

INTRODUCTION

Many proteins involved in cell signalling are modified by lipidation and methylation [1–3]. These modifications are essential for the correct association of these proteins with their target membrane and/or their interactions with other proteins [3,4]. The γ-subunit of heterotrimeric G-proteins and many members of the superfamily of small ras-related G-proteins are prenylated at their C-terminal cysteine followed by their carboxymethylation [1–3].

Two types of prenyl group, i.e. farnesyl and geranylgeranyl, are transferred by different transferases to the C-terminal cysteine of G-proteins through a thioether linkage. For those proteins with a CAAX (where C stands for cysteine, A is an aliphatic amino acid and X is any amino acid) motif a common methyltransferase may be responsible for the methylation [2,5], whereas a different methyltransferase is specific for proteins (e.g. some rab proteins) having CXC motifs [6]. Prenylation is a relatively stable co- (or immediately post-) translational reaction and may be irreversible. In contrast, there is evidence that methylation of carboxyprenylcysteine can be stimulated, as well as being reversible ([1,2]; A. Kowluru, G. Li and S. A. Metz, unpublished work) and thus could act as a switch for protein function in cell activation.

Prenylcysteine carboxymethyltransferase (protein-*S*-isoprenylcysteine *O*-methyltransferase; EC 2.1.1.100) activity has been identified in many cellular systems [2,5,7–10]. This enzyme catalyses the methylation not only of native proteins but also of short peptides of farnesylcysteine as well as farnesylcysteine analogues [5]. The ability of the enzyme to methylate both farnesyl- and geranylgeranyl-cysteine and the cross-competitive

inhibition between the two cysteine analogues suggest the involvement of only one type of methyltransferase acting on the CAAX motif [2].

The gene for the farnesylcysteine carboxymethyltransferase in yeast has been cloned; its mutant displayed an impaired protein–protein interaction and aberrant transport of α-factor [5]. It has been reported that carboxymethylation is involved in the activation of platelets, neutrophils and macrophages [1,2,5] as well as of normal rat islets [11,12] and cloned β (βTC) cells [10]. Blockade of protein carboxymethylation using certain competitive inhibitors impeded chemotactic responses [2,5,13] and insulin secretion [10,11]; both heterotrimeric and small G-proteins have been implicated in these events [2,5]. Prenylcysteine carboxymethylation is also important in visual transduction [14] and may be involved in the activation of phospholipase C and phosphoinositide 3-kinase [15]. In insulin-secreting cells, interference with prenylation and methylation impaired insulin release during stimulation [11,16]. More recently, it has been found that methylation of the ras-related small G-proteins rap1 [10] and CDC42 [12] may be involved in the regulation of insulin secretion.

The aim of the present study was to characterize the prenylcysteine carboxymethyltransferase activity in an insulin-secreting cell line, INS-1, which possesses many of the properties of primary pancreatic β-cells [17]. The results revealed that the enzyme is tightly bound to the cellular membranes, with highest specific activity in the plasma membrane and probably also in the endoplasmic reticulum. This methyltransferase seems not to be regulated by conventional protein kinases and may be constitutively active, suggesting that the regulation of carboxymethylation is at the level of substrate activation.

Abbreviations used: AFC, *N*-acetyl-*S*-trans,trans-farnesyl-L-cysteine; AdoMet, *S*-adenosyl-L-methionine; ER, endoplasmic reticulum; GDI, GDP-dissociation inhibitor; GDS, GDP-dissociation stimulator; GTP[S], guanosine 5'-[γ-thio]triphosphate; PMA, phorbol 12-myristate 13-acetate.

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EXPERIMENTAL

Materials

N-Acetyl-*S*-*trans,trans*-farnesyl-L-cysteine (AFC) and *N*-acetyl-*S*-*trans,trans*-geranylgeranyl-L-cysteine were purchased from Biomol, Plymouth Meeting, PA, U.S.A.; *S*-adenosyl-L-[methyl-³H]methionine (Ado[³H]Met) and [γ -³²P]ATP were from du Pont NEN, Boston, MA, U.S.A.; Percoll, cAMP, mastoparan and phorbol 12-myristate 13-acetate (PMA) were from Sigma, St. Louis, MO, U.S.A.; arachidonic acid was from Nu-Chek-Prep, Elysian, MN, U.S.A. Sinefungin was a gift from Eli Lilly Co., Indianapolis, IN, U.S.A.

INS-1 cell culture and isolation of islets

INS-1 cells, a gift from Dr. C. B. Wollheim (University of Geneva, Switzerland), were cultured (between passages 48 and 79) in RPMI 1640 containing 2-mercaptoethanol and pyruvate as described in detail previously [17]. Rat pancreatic islets were isolated and homogenized as described in [18].

INS-1 cell homogenization and subcellular fractionation

All procedures were performed on ice or in a 4 °C cold room. Cells (about 600×10^6) were washed twice with cold PBS and scraped into homogenization buffer containing 20 mM Tris/HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol and the following protease inhibitors: leupeptin (10 μ g/ml), aprotinin (4 μ g/ml), pepstatin (2 μ g/ml) and PMSF (100 μ M). The cells were then disrupted either by sonication (2 $\times$ 10 s) on ice or by mechanical homogenization (1000 rev./min; 10 strokes), which was repeated once or twice to the sediments after centrifugation.

For separation of total particulate and soluble fractions, the homogenates were centrifuged for 60 min at 100 000 *g* and the pellets were resuspended in homogenization buffer (about 1 μ g of protein/ μ l).

For subcellular fractionation, the homogenates obtained by mechanical homogenization were first centrifuged at 900 *g* to remove the nuclei and unbroken cells. The post-nuclear supernatant was then centrifuged at 5500 *g* for 15 min, 25 000 *g* for 20 min and 100 000 *g* for 60 min, to obtain putative mitochondria-enriched, secretory-granule-enriched and microsomal fractions respectively, in a Beckman Optima TL ultracentrifuge. The subcellular content of these fractions has been previously characterized extensively using rat islets [18]. In order to achieve a better separation, the mitochondria- and secretory-granule-enriched fractions were subjected to density centrifugation in 30% Percoll using the protocol detailed in [19]. For separation of the endoplasmic reticulum (ER) from the plasma membrane, centrifugation in sucrose gradients (1.14, 1.16, 1.18 and 1.20 g/ml) was performed as described in [20].

Protein was measured by the Bio-Rad assay. All samples were immediately used for enzyme assay or stored at -70 °C.

Prenylcysteine methyltransferase assay

The activity of farnesylcysteine carboxymethyltransferase was measured using AFC as substrate; this forms a base-labile, hydrophobic, methyl ester in the presence of Ado[³H]Met, as described elsewhere [13]. Assay buffer (25 μ l; containing 100 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 10 μ M unlabelled AdoMet, approx. 1 μ Ci of Ado[³H]Met and 200 μ M AFC) was mixed with 25 μ l samples and incubated at 37 °C for 30 min except where indicated. The AFC methyl ester formed

was extracted by adding 0.5 ml of chloroform/methanol (1:1, v/v) and 0.25 ml of water followed by vigorous vortex-mixing. The organic phase (125 μ l) was transferred to an Eppendorf tube and dried at 40 °C in a ventilation hood. After addition of 100 μ l of 1 M NaOH containing 1% SDS, each tube was carefully placed in a vial containing scintillation solution and capped well. The vials were left at 37 °C for 24 h to allow equilibration of the evaporated [³H]methyl vapour in the scintillation solution before counting in a β -radiation counter.

Marker enzyme assays

The following marker enzymes were measured: 5'-nucleotidase [21] and ouabain-sensitive Na⁺/K⁺-ATPase [22] for plasma membranes, cytochrome *c* oxidase for mitochondria [23], NADPH:cytochrome *c* reductase [24] and glucose-6-phosphatase [21,25] for ER, and aryl sulphatase for lysosomes [26]. Insulin content, determined by RIA, was used as the secretory granule marker.

Statistics

The results were expressed as means $\pm$ S.E.M. and analysed using Student's unpaired two-tailed *t* test.

RESULTS

Localization of prenylcysteine carboxymethyltransferase

Homogenates either from isolated pancreatic islets or from a cultured insulin-secreting cell line (INS-1) equipotently catalysed

Figure 1 Protein dose-response (a) and time course (b) of the activity of prenylcysteine carboxymethyltransferase in INS-1 cell homogenates

The values are means $\pm$ S.E.M. of three observations. The error bars are not visible in some cases because they fall within the symbols.

Figure 2 Kinetics of prenylcysteine carboxymethyltransferase in INS-1 cell homogenates

The values are means $\pm$ S.E.M. of three observations. The inset shows the Lineweaver-Burk double-reciprocal plot of AFC methyl ester formation versus AFC concentration by regression analysis, which gave the estimated values of the K_m (x -axis intercept is $-1/K_m$) and V_{max} (y -axis intercept is $1/V_{max}$) of the enzyme for AFC.

the methylation of AFC. Since a large amount of protein is needed for the enzyme assay (approx. 20 μ g per tube), the INS-1 cells were used to characterize the methyltransferase in all subsequent studies. INS-1 cell homogenates promoted the methylation of AFC in a dose- and time-dependent manner (Figure 1). The K_m of the methyltransferase for AFC was estimated as 30.5 ± 3.5 μ M ($n = 3$), with a V_{max} of 3.5 ± 0.1 pmol/min per mg of protein (Figure 2). The β -cell methyltransferase could also use *N*-acetyl-*S*-*trans*,*trans*-geranylgeranyl-L-cysteine as substrate, with a K_m (approx. 10 μ M) lower than that for AFC (results not shown).

The farnesylcysteine carboxymethyltransferase was inhibited by two general methylation inhibitors, *S*-adenosylhomocysteine and sinefungin [27] (Figure 3). The latter (EC_{50} approx. 0.9 μ M) was more potent than the former (EC_{50} approx. 20 μ M).

The distribution of the methyltransferase was first examined in the subcellular fractions obtained by differential centrifugation. As shown in Table 1, most enzyme activity was found in the 5500 g and 25000 g pellets, with some in the 100000 g pellet and less than 5% of the total activity in the cytosol, indicating that the enzyme was largely associated with the particulate fractions. To assess how tightly the enzyme is associated with the membranes, the homogenates were extracted with 3 M NaCl or KCl for 60 min at 4 $^{\circ}$ C followed by a 90 min centrifugation at 100000 g to separate the particulate and the soluble fractions. In such high-salt-extracted samples, the activity of the prenylcysteine methyltransferase in the membranes was reduced from 94% to 60% of the total activity, in comparison with the marked decrease in protein in the particulate fraction from 60% to 16% of the total, indicating the most of that enzyme is tightly bound to membranes.

The data in Table 1 also reveal that the highest specific activity (7.5 times that in the post-nuclear supernatant) was present in the 25000 g pellet, which is enriched in insulin granules (Table 2) [18,28]. The 5500 g pellet exhibited a modest enrichment in specific activity (3.7-fold), while the 100000 g pellet displayed a very low methyltransferase specific activity.

Figure 3 Inhibition of prenylcysteine carboxymethyltransferase in INS-1 cell homogenates by *S*-adenosylhomocysteine and sinefungin

The values are mean $\pm$ S.E.M. of three observations.

Enzyme markers for cellular organelles were quantified in the subcellular fractions obtained by differential centrifugation in order to define further the localization of the majority of the methyltransferase activity; the results are summarized in Table 2. The activities of cytochrome *c* oxidase (a mitochondrial marker) and aryl sulphatase (a lysosomal marker) were highest in the 5500 g pellet, whereas only a small fraction of the former and even less of the latter were detected in the 25000 g pellet, indicating a poor co-localization of these two organelles with the methyltransferase. In the 5500 g and 25000 g pellets there was a good correlation between the activity of the methyltransferase and the insulin content, the plasma membrane markers (e.g. 5'-nucleotidase) and the ER markers (e.g. NADPH reductase). Comparable specific activities of glucose-6-phosphatase, which is also an ER marker, were detected in both the 5500 g and 25000 g pellets. Surprisingly, the 100000 g pellet (which is the putative microsomal fraction based on studies of rat islets) also exhibited rather low activities of both the plasma membrane markers and the ER markers examined, suggesting that the microsomal fraction might in fact contain substantial methyltransferase activity.

Electron microscopy of the subcellular fractions obtained by differential centrifugation reflected the results of measurements of subcellular organelle markers. The 5500 g pellet contained significant amounts of mitochondria and secretory granules. Some contaminating lysosomes and other heterogeneous membrane structures were also visible. The 25000 g pellet contained many insulin granules and also a large amount of heterogeneous membrane structures, including some ER but not mitochondria and only a few lysosomes. The 100000 g pellet was mainly composed of cell debris and unidentified subcellular structures (results not shown).

These results raised the possibility that the methyltransferase is enriched in the insulin-secretory granules. To address this point, density centrifugation of the pellets from the 25000 g fraction was performed using pre-formed Percoll gradients [19]. This technique yielded a highly purified (> 20 -fold) fraction of insulin-containing granules and a mixture of the ER- and plasma

Table 1 Distribution of prenylcysteine methyltransferase (MT) activity in relation to protein content in subcellular fractions of INS-1 cells obtained after differential centrifugation

The values are means $\pm$ S.E.M. from six independent experiments. MT distribution is presented by giving the total methyltransferase activity in each fraction as a percentage of the total enzyme activity in the post-nuclear supernatant originally used for fractionation.

	Post-nuclear fraction	5500 <i>g</i> pellet	25 000 <i>g</i> pellet	100 000 <i>g</i> pellet	Cytosol	Recovery (%)
MT activity (pmol/min per mg of protein)	1.86 $\pm$ 0.18	3.74 $\pm$ 0.27	7.45 $\pm$ 0.5	0.91 $\pm$ 0.29	0.10 $\pm$ 0.02	
MT distribution (% of post-nuclear)	100	36.7 $\pm$ 3.4	31.0 $\pm$ 3.2	9.0 $\pm$ 2.8	3.8 $\pm$ 0.7	80.5 $\pm$ 4.4
Protein content (% of post-nuclear)	100	18.1 $\pm$ 2.5	7.2 $\pm$ 0.8	15.8 $\pm$ 0.9	62.6 $\pm$ 2.3	103.7 $\pm$ 3.2

Table 2 Activity of farnesylcysteine methyltransferase and of enzymes markers for subcellular organelles in subcellular fractions of INS-1 cells after differential centrifugation

The values are mean $\pm$ S.E.M.; the numbers of independent experiments are indicated in parentheses.

Enzyme activity or insulin content	Post-nuclear fraction	5500 <i>g</i> pellet	25 000 <i>g</i> pellet	100 000 <i>g</i> pellet	Cytosol
Methyltransferase (pmol/min per mg of protein)	2.58 $\pm$ 0.63 (7)	7.01 $\pm$ 1.47 (7)	12.74 $\pm$ 3.05 (7)	1.26 $\pm$ 0.28 (7)	0.08 $\pm$ 0.01 (7)
Cytochrome <i>c</i> oxidase (μ mol/min per mg of protein)	0.34 $\pm$ 0.08 (6)	1.35 $\pm$ 0.22 (6)	0.17 $\pm$ 0.07 (6)	0.04 $\pm$ 0.01 (5)	0.06 $\pm$ 0.01 (3)
Aryl sulphatase (nmol/min per mg of protein)	11.13 $\pm$ 2.79 (5)	45.66 $\pm$ 4.72 (5)	33.64 $\pm$ 5.53 (5)	1.25 $\pm$ 0.31 (5)	2.68 $\pm$ 0.42 (3)
Insulin content (μ g/mg of protein)	3.31 $\pm$ 0.78 (7)	8.64 $\pm$ 1.74 (5)	11.68 $\pm$ 1.43 (7)	0.33 $\pm$ 0.17 (7)	0.03 $\pm$ 0.02 (7)
NADPH reductase (nmol/min per mg of protein)	110 $\pm$ 17 (7)	279 $\pm$ 50 (7)	470 $\pm$ 85 (7)	41 $\pm$ 10 (7)	14 $\pm$ 11 (3)
Glucose-6-phosphatase (nmol/min per mg of protein)	10.18 $\pm$ 0.89 (4)	26.40 $\pm$ 1.85 (4)	25.37 $\pm$ 3.78 (4)	1.71 $\pm$ 0.38 (4)	2.56 $\pm$ 0.28 (4)
5'-Nucleotidase (nmol/min per mg of protein)	13.42 $\pm$ 1.36 (4)	38.96 $\pm$ 3.24 (4)	45.31 $\pm$ 4.91 (4)	4.82 $\pm$ 1.26 (4)	2.44 $\pm$ 0.58 (4)

Table 3 Activity of farnesylcysteine methyltransferase and of enzyme markers for subcellular organelles in the subcellular fractions of INS-1 cells after Percoll density gradient centrifugation

The 25 000 *g* pellet from differential centrifugation was resuspended and loaded on to 30% Percoll which had been pre-centrifuged at 22 000 *g* for 5 min to form a density gradient. The sample-loaded tubes were centrifuged at 9000 *g* for 60 min using rotor SW41Ti in a Beckman L3-50 ultracentrifuge. The top 25% and bottom 25% portions were transferred to clean tubes and washed three times at 24 000 *g*. Results are from three independent experiments. Values are means $\pm$ S.E.M., and the numbers of determinations are indicated in parentheses. n.d., not determined due to a limited amount of protein.

Enzyme activity or insulin content	Top fraction	Bottom fraction
Methyltransferase (pmol/min per mg of protein)	16.91 $\pm$ 4.98 (3)	0.41 $\pm$ 0.27 (3)
Insulin content (μ g/mg of protein)	19.90 $\pm$ 4.60 (3)	74.40 $\pm$ 3.46 (3)
NADPH reductase (nmol/min per mg of protein)	527 $\pm$ 132 (3)	30 (2)
Glucose-6-phosphatase (nmol/min per mg of protein)	35.61 $\pm$ 4.37 (3)	97.20 (2)
5'-Nucleotidase (nmol/min per mg of protein)	75.12 $\pm$ 10.72 (3)	n.d.

membrane-enriched fractions (Table 3). Methyltransferase activity was barely detectable in this highly purified insulin granule fraction, whereas in the ER- and plasma membrane-enriched fractions the specific activity of methyltransferase was increased by about 50% over that in the 25 000 *g* pellet. This correlated well with the markers of these two subcellular structures, determined in the same fractions (Table 3). Thus these results largely exclude the possibility of an enrichment of the methyltransferase in the insulin granules. Furthermore, since glucose-6-

phosphatase was also enriched in the insulin-granule-enriched fraction, it seems unlikely that the ER is a major site of localization of the methyltransferase. However, these data do not exclude the possibility that a subfraction of the ER, which is deficient in glucose-6-phosphatase, contains the methyltransferase. Additionally, one cannot totally exclude the possibility that the methyltransferase is inactivated in the environment of secretory granules (i.e. high Zn^{2+} content; low pH).

Therefore, we next attempted to separate the plasma membrane and the ER by sucrose density centrifugation, an approach which has been used previously with pancreatic islets [20]. Four bands between the interfaces (I, II, III and IV, from top to bottom) were obtained after this procedure. The highest methyltransferase specific activity was found in band I, with moderate activity in band II. Band I contained the highest specific activity of both of the plasma membrane markers 5'-nucleotidase and Na^+/K^+ -ATPase, as reported in [20]. However, band I also exhibited high NADPH reductase activity, compatible with findings of others using insulinoma tissue [19]. The activity of glucose-6-phosphatase was distributed in all four bands, and again was not correlated with NADPH reductase activity (results not shown). Thus these results indicate a close correlation between the activities of the methyltransferase and of the plasma membrane markers; however, the possibility of methyltransferase activity in a subfraction of the ER could not be excluded (see the Discussion section).

Regulation of carboxymethylation

It has been reported that prenylcysteine carboxymethylation is implicated in cell activation [1,2,5,10,11,14,15] and that GTP analogues stimulate the methylation of certain ras-related G-proteins [2,8]. Furthermore, we have observed that the methylation of CDC42 is stimulated by glucose but not by

Table 4 Farnesylcysteine methyltransferase (MT) activity in INS-1 cells homogenates under various conditions

INS-1 cell homogenates were incubated in methyltransferase assay buffer supplemented with the indicated agents for 30 min. In the Ca^{2+} -treated group, 0.25 mM Ca^{2+} in excess of EDTA (0.5 mM) and EGTA (0.25 mM) was added. Values are means $\pm$ S.E.M., with numbers of determinations in parentheses.

Condition	MT activity (pmol/min per mg of protein)
Control	1.50 $\pm$ 0.09 (11)
Ca^{2+} (250 μM)	1.61 $\pm$ 0.03 (6)
cAMP* (100 μM)	1.41 $\pm$ 0.07 (5)
PMA* (100 nM)	1.43 $\pm$ 0.07 (11)
GTP[S] (100 μM)	1.56 $\pm$ 0.09 (6)
Mastoparan (30 μM)	1.39 $\pm$ 0.08 (6)

* 2.5 mM ATP and 3 mM Mg^{2+} were also included.

40 mM KCl in intact islets and HIT cells (A. Kowluru, G. Li and S. A. Metz, unpublished work). Therefore the possibility that the methyltransferase activity is regulated was examined both in broken cell and in intact cells.

In cell-free preparations (Table 4), guanosine 5'-[γ -thio]-triphosphate (GTP[S]) (100 μM) did not increase methyltransferase activity, although we and others have reported that GTP[S] promotes the methylation of small G-proteins [2,11,12]. These findings suggest that the stimulation of G-protein methylation by GTP[S] may be due to its effect on the conformation of G-proteins, induced by its binding, rather than a direct action on the methyltransferase. Mastoparan, a potent stimulator of most G-proteins [29] and an initiator of insulin release [18,30], did not alter methyltransferase activity. The effects of three conventional second messengers in cell activation (Ca^{2+} , the protein kinase A activator cAMP and the protein kinase C stimulator PMA) on the methyltransferase were also investigated. None of them appeared to alter enzyme activity. Additionally, in INS-1 cells pre-stimulated with various secretagogues for insulin release (15 mM glucose alone or combined with forskolin, or 40 mM K^+) [17], there was no change in the enzyme activity in homogenates or in cytosol and membrane fractions (G. Li, A. Kowluru and S. A. Metz, unpublished work). Finally, neither Ca^{2+} nor cAMP promoted the carboxymethylation of CDC42 (in the presence or absence of GTP[S]) in β -cell homogenates (results not shown). Taken together, these observations suggest that prenylcysteine carboxymethyltransferase is not regulated by conventional kinase pathways.

It has been reported that arachidonic acid promotes the translocation of small G-proteins to membranes [31]; the underlying mechanism is not clear. However, we have observed that it may promote formation of the GTP-liganded form of G-proteins [18]. Since methylation is required for maximal membrane association of GTP-binding proteins [2,32], the effect of this fatty acid on the activity of prenylcysteine carboxymethyltransferase was examined. Arachidonic acid actually inhibited the enzyme activity in a dose-dependent manner (20–330 μM) with an EC_{50} of approx. 80 μM (G. Li, A. Kowluru and S. A. Metz, unpublished work), which is within the range of concentrations that induce insulin release from pancreatic β -cells [33]. This inhibition of methyltransferase by arachidonic acid was not altered by indomethacin (200 μM), an inhibitor of arachidonic acid metabolism via cyclo-oxygenase. However, the effect of arachidonic

acid on the methyltransferase is not specific, since other unsaturated fatty acids such as oleic acid and linoleic acid (but not stearic acid) also inhibited the methyltransferase (results not shown). The significance of the inhibitory effect of arachidonic acid on the methyltransferase is currently unclear; however, arachidonate also decreased the carboxymethylation of CDC42 and of the γ -subunit of heterotrimeric G-proteins in HIT cell homogenates (results not shown). Additionally, our study indicates that promotion of the translocation of small G-proteins to the membranes by arachidonic acid [31] is apparently not due to an effect on the methyltransferase itself.

We have reported that AFC decreases carboxymethylation of low-molecular-mass G-proteins in normal rat islets; concomitantly, AFC reduces nutrient-, but not K^+ -, induced insulin release [11]. Therefore we extended these studies to INS-1 cells and obtained similar results. Thus, similar to our previous observations using isolated islets [11], when INS-1 cells were pretreated with 100 μM AFC for 60 min the insulin secretion induced by 10 mM glucose alone or glucose plus forskolin (to increase cAMP) was inhibited, whereas the effect of high K^+ (to increase the cytosolic free Ca^{2+} concentration) on the stimulation of insulin release was not affected (results not shown). This suggests that carboxymethylation does not regulate the distal step(s) in hormone exocytosis.

DISCUSSION

Previous data provide evidence for a role for the modification of proteins by methylation and demethylation of their α -carboxy groups, similar to phosphorylation and dephosphorylation, in the regulation of protein function [1,2,4]. A large number of signalling proteins, such as the γ -subunit of heterotrimeric G-proteins and many ras-related small GTP-binding proteins, undergo a series of consecutive modifications at their C-terminal cysteine, including isoprenylation, proteolytic removal of last three amino acids, and carboxymethylation. These modifications increase protein hydrophobicity and change protein conformation, permitting the selective association of the proteins with their target membranes and their interaction with regulatory and/or effector proteins [3,4,34]. Although isoprenylation may be sufficient for some proteins to bind to membranes or proteins [3], methylation, which is reversible and thus might be regulated, is also required for the binding of other proteins and/or for function [2,14,35]. There is evidence for a role for prenylcysteine carboxymethylation in the activation of platelets, neutrophils and macrophages, and in the regulation of insulin secretion [2,5,10,11]. A transient increase in the carboxymethylation of small G-proteins is observed in stimulated neutrophils [36] and pancreatic β -cells [10], and inhibitors of the methylation such as AFC impair the functional responses in these cells ([5,11,13]; the present study). AFC competitively inhibits the prenylcysteine carboxymethylation of the endogenous proteins that are involved in the regulation of hormone secretion and other functions [5]. Thus knowledge of the enzyme that catalyses methylation is important for our understanding of this signalling event.

Our results reveal that more than 95% of the prenylcysteine carboxymethyltransferase activity in INS-1 cells is associated with the total particulate fraction (presumably the membranes), an observation concurring with the findings in other cell systems [2,5,7,8,13]. The K_m of this enzyme for AFC, and its inhibition by methylation inhibitors, also indicate that the methyltransferase in INS-1 cells is similar to those found in other cells [2,9]. The extraction of INS-1 cell homogenates with high salt concentrations removed only about one-third of the membrane-bound

methyltransferase activity, suggesting a tight association of the enzyme with the membranes. Interestingly, this enzyme activity extracted from membranes was fully recovered in the soluble fraction, indicating that the methyltransferase may not need a membrane environment for its activity. However, it is possible that the salt-extracted methyltransferase is just one of several isoforms. In view of the high sensitivity of the methyltransferase to detergents [21,37] and the poor recovery of its activity after detergent removal [37], high-salt extraction may be an alternative method for the isolation and purification of the enzyme.

The subcellular distribution of prenylcysteine carboxymethyltransferase is debated. The enzyme was found mainly in the plasma membrane in neutrophils [37], whereas the highest methyltransferase activity was reported to be in the ER of hepatocytes [21]. In the kidney cortex, enriched methyltransferase activity was detected both in the plasma membrane- and in the ER-enriched fractions [8]. Our differential centrifugation studies (Table 1) initially suggested that methyltransferase activity might be enriched in the secretory granule fraction. Considerable enzyme activity was also detected in a similar fraction in another insulin-secreting cell line, β TC [10]. However, true enrichment of the enzyme in the β -cell secretory granules was excluded after purification of the granules by Percoll density centrifugation. We also did not detect methyltransferase activity in the purified granule fraction from rat insulinoma tissue (provided by Dr. C. J. Rhodes, Boston, MA, U.S.A.; results not shown). The analyses of the co-localization of the methyltransferase and the marker enzymes of subcellular organelles also indicated low methyltransferase activity in mitochondria and lysosomes. Our results reveal that the methyltransferase activity was best correlated with the marker enzymes for the plasma membrane. However, the presence of significant methyltransferase activity in the ER could not be totally excluded, since we have not separated the two cross-contaminated membranes completely. Our studies also do not exclude the presence of the enzyme in other subcellular membranes such as nuclei, Golgi complex and synaptic-like microvesicles, especially considering that methylated small GTP-binding proteins are found in these organelles [3,4].

Glucose-6-phosphatase is believed to be a marker enzyme for the ER [21], and its presence in pancreatic islets is still under debate [38,39]. In the present study using INS-1 cells the activity of this enzyme was not correlated with that of another ER marker enzyme, NADPH reductase. Furthermore, considerable glucose-6-phosphatase activity was detected in a highly purified granule-enriched fraction after Percoll density centrifugation. It seems that glucose-6-phosphatase is localized either in a distinct organelle different from the ER or, more likely, in a particular subcompartment of the ER that lacks NADPH reductase activity. It is tempting to speculate that such a compartment might be the compartment recently described in studies of rat liver homogenates [40] and neonatal β -cells [41], in which a plasma membrane fraction seems to be closely associated with the smooth ER.

Several low-molecular-mass small G-proteins, e.g. rab3, rap1 and CDC42, might be involved in the regulation of exocytosis from vesicles/granules containing neurotransmitters/hormones, including insulin [10–12,16,42]. An increase in the carboxymethylation of several G-proteins upon cell activation [2,10,11,13,16] raises the possibility that cell activation augments methyltransferase activity, perhaps mediated by a signalling pathway such as a Ca^{2+} -, cAMP- or protein kinase C-mediated process. Using AFC as substrate, we failed to observe an effect of Ca^{2+} , cAMP or PMA (a potent protein kinase C activator) on methyltransferase activity *in vitro*. Similarly, stimulation of intact cells for 30 min by glucose, high K^+ , forskolin and PMA did not

stably modify enzyme activity or distribution. However, due to technical restrictions, we did not measure methyltransferase activity after short cell incubations (10–20 s), the time correlated with the peak rise in methylation observed in intact neutrophils and insulin-secreting cells ([10]; A. Kowluru, G. Li and S. A. Metz, unpublished work). GTP[S], which is able to stimulate the methylation of small G-proteins [2,37], also failed to increase methyltransferase activity directly, in agreement with other studies [2,8]. Mastoparan, a peptide with potent ability to stimulate G-proteins, also did not alter methyltransferase activity directly. This observation concurs with the report that the receptor agonist f-Met-Leu-Phe, which activates G-proteins and enhances methylation in neutrophils, had no direct effect on the methyltransferase [37]. Therefore it is likely the methyltransferase is in a constitutively active state and that other steps that control the activation of G-proteins or their accessibility to the methyltransferase regulate their carboxymethylation. The binding of GTP may regulate the methylation of G-proteins *in vivo* by increasing their affinity for the methyltransferase [36,37]. These possibilities could not be examined in the current studies, since AFC is an artificial substrate which does not bind GTP.

It is well established that cycling between an active (GTP-bound) state and an inactive (GDP-bound) state of low-molecular-mass G-proteins is regulated by several proteins [3,4]. GDP-dissociation inhibitor (GDI) inhibits, and GDP-dissociation stimulator (GDS) activates, G-proteins. The inactive form of G-proteins is 'trapped' in the cytosol by GDI. Since prenylation but not methylation is required for the interaction of GTP-binding proteins with GDI [3,43], it seems likely that GDI masks the C-terminus of G-proteins, impeding their methylation. However, when the G-proteins are activated (i.e. application of GTP[S] or stimulation by GDS), binding by GTP or its analogues can promote methylation. Indeed, it has been reported that GDI regulates the carboxymethylation of CDC42 [44] and, conversely, that GTP[S] promotes the association of GTP-binding proteins with the membrane and increases their affinity for the methyltransferase [2]. The physiological significance of G-protein methylation could be to reinforce their association with membranes, and to ensure their correct targeting to their molecular effectors. The hydrolysis of GTP promoted by a GTPase-activating protein could render the methylated G-proteins inactive, and the GDP-bound proteins thus would dissociate from membranes, due to the action of GDI. Little is known about how methylated G-proteins are demethylated, but methylesterases are found both in the cytosol and in membranes [2]. Methylesterase activity has also been detected in insulin-secreting cells [45]. AFC methyl ester, however, is barely hydrolysed by INS-1 cell homogenates, at least within 30 min (results not shown); therefore the conformation of a native protein may be required for its recognition by the methylesterase.

In conclusion, the prenylcysteine carboxymethyltransferase in insulin-secreting cells is a membrane-bound enzyme. Based on analyses of marker enzymes, methyltransferase activity appears to be predominantly associated with the plasma membrane and possibly also the ER. The enzyme activity does not seem to be increased directly by conventional kinases or cellular messengers. The extant data suggest that one important factor in the regulation of G-protein methylation is the conformation of the G-proteins which, in turn, is controlled by GTP and regulatory proteins. Since glucose increases the methylation of low-molecular-mass G-proteins *in vivo* without stably modifying the methyltransferase, it appears tenable that glucose may alter the carboxymethylation of key β -cell proteins by modifying GTP synthesis and/or the exchange of GTP for GDP on G-proteins. Further studies are required to address this hypothesis.

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