Characterization of Two Recombinant PDE3 (cGMP-Inhibited Cyclic Nucleotide Phosphodiesterase) Isoforms, RcGIP1 and HcGIP2, Expressed in NIH 3006 Murine Fibroblasts and Sf9 Insect Cells†

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ABSTRACT: cDNAs encoding PDE3 [cGMP-inhibited cyclic nucleotide phosphodiesterase (cGI PDE)] isoforms, cGIP1 and cGIP2, have been cloned from rat (R) and human (H) cDNA libraries. The deduced amino acid sequences of RcGIP1 and HcGIP2 are very similar in their conserved catalytic domains but differ in their N-terminal regulatory domains [Meacci, E., et al. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3721−3725; Taira, M., et al. (1993) *J. Biol. Chem. 268*, 18573−18579]. cDNAs encoding both rat adipocyte RcGIP1 and human myocardial HcGIP2 (full-length forms and truncated forms lacking much of the putative N-terminal domain) were expressed in NIH 3006 fibroblasts and in Sf9 insect cells. The recombinant proteins exhibited the expected subunit molecular mass, immunologic reactivities, and characteristics of native membrane-associated forms of the enzymes, e.g., high affinity for cAMP (K_m), sensitivity to the selective cGI PDE inhibitors OPC 3689 and OPC 3911 and to cGMP. The full-length recombinants were predominantly particulate, whereas the truncated HcGIP2 forms were cytosolic suggesting that N-terminal domains contain structural determinants important for membrane association. Both fibroblast RcGIP1 and authentic adipocyte cGI PDE were phosphorylated *in vitro* by cAMP-dependent protein kinase; tryptic [^{32}P]peptides released from rat adipocyte ^{32}P-cGI PDE and ^{32}P-RcGIP1 exhibited identical electrophoretic profiles suggesting that the same peptides are phosphorylated in both.

By catalyzing hydrolysis of cyclic nucleotides, cyclic nucleotide phosphodiesterases (PDEs) are critical regulators of the intracellular concentrations and biological effects of these important second messengers. PDEs constitute a large superfamily of enzymes composed of at least seven different gene families (PDE1−7) that have been defined on the basis of differences in primary amino acid sequences, catalytic, immunological and structural characteristics, responses to specific effectors and inhibitors, and regulatory mechanisms (Beavo et al., 1994; Manganiello et al., 1995).

The PDE3, or cGMP-inhibited phosphodiesterase (cGI PDE) family (Beavo et al., 1994), is involved in regulation of several physiological processes (Manganiello et al., 1990). Activation of a microsomal cGI PDE in fat cells is important in the antilipolytic action of insulin (Degerman et al., 1996). Specific cGI PDE inhibitors promote smooth muscle (especially airway and vascular) relaxation, enhance myocardial contractility, and inhibit platelet aggregation (Beavo & Reifsnyder, 1990; Thompson, 1991; Nicholson et al., 1991). cGI PDEs are found in cytosol and in association with intracellular membranes (Manganiello et al., 1990).

We have cloned cDNAs which encode adipocyte (cGIP1) and myocardial (cGIP2) isoforms (corresponding to PDE3B and PDE3A, respectively; Beavo et al., 1994) from human- (H) and rat(R) cDNA libraries; the two cGI PDE isoforms are products of distinct, but related genes. RcGIP1 and HcGIP1 (or RcGIP2 and HcGIP2) are more similar to each other than are RcGIP1 and RcGIP2 (or HcGIP1 and HcGIP2) (Meacci et al., 1992; Taira et al., 1993, unpublished data). Both isoforms contain the catalytic domain conserved among all mammalian PDEs in the C-terminal region (Charbonneau, 1990) and, near the N-terminus, large hydrophobic regions containing multiple predicted transmembrane segments (Meacci et al., 1992; Taira et al., 1993). These putative membrane-association domains and conserved catalytic domains are separated by putative regulatory domains, which contain several consensus sequences for phosphorylation by cAMP-dependent protein kinase (cAMP-PrK) (Meacci et al., 1992; Taira et al., 1993). Based on the deduced sequence of RcGIP1, serine 427 is the major site for phosphorylation of the solubilized adipocyte microsomal cGI PDE by cAMP-

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PrK *in vitro* (Rasçon et al., 1994). *In situ* hybridization studies indicate that, in the rat, cGIP1 and cGIP2 mRNAs are expressed in different tissues, raising the possibility of tissue-specific regulation and functions for cGIP1 and cGIP2 (Reinhardt et al., 1995).

In the present study, cDNAs encoding rat adipocyte RcGIP1 and human myocardial HcGIP2 (both a "full-length" form including the entire open reading frame and truncated forms lacking much of the putative N-terminal regulatory and membrane-association domains) have been expressed and characterized, and found to have properties similar to those of native membrane-associated cGI PDE isoforms. The full-length recombinants were predominantly particulate, whereas the truncated HcGIP2 forms were cytosolic, suggesting that the hydrophobic N-terminal domains of cGI PDEs contain structural determinants important for membrane association.

MATERIALS AND METHODS

Expression of RcGIP1 and HcGIP2 in NIH 3006 Fibroblasts

Construction of Vectors. RcGIP1 cDNA, subcloned into pBluescript II (KS+) (Stratagene) (M. Taira, unpublished data), was excised with *Spe*1. Blunted ends were constructed with Klenow enzyme [EXO(−), Prime-It II Random Primer Labeling Kit, Stratagene]. An *Eco*R1 restriction fragment (∼4 kb) containing the entire open reading frame of HcGIP2 cDNA (Meacci et al., 1992) was subcloned into pBluescript II (SK+) and excised with *Xho*1 and *Not*1. pBPV, a bovine papilloma virus-derived mammalian expression vector (Pharmacia LKB Biotechnology, Inc.), was digested with *Not*1 for blunt end ligation with RcGIP1 or *Xho*1/*Not*1 for ligation with HcGIP2.

RcGIP1 and HcGIP2 cDNAs were subcloned into pBPV, amplified, and purified, and the nucleotide sequences of their 5′ and 3′ ends were confirmed by the dideoxynucleotide method using Sequenase version 2.0 (United States Biochemical).

Cell Culture and Transfection. NIH 3006 fibroblasts, NIH 3T3 fibroblasts which stably overexpress human insulin receptors (Cama et al., 1992), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin−streptomycin−fungizone in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Plasmid cDNAs were purified by equilibrium centrifugation in CsCl (Sambrook et al., 1989). Expression plasmids or pBPV without cDNA insert (20 μg) and PY3 vector (1 μg) (hygromycin resistance plasmid) were cotransfected using calcium phosphate precipitation (Ausubel et al., 1993) in 100 mm culture dishes for 18 h at 37 °C with 3% CO_2. Cells were harvested with trypsin (2 mL of 0.1% trypsin/0.02% EDTA), dispensed into 150 mm dishes, and incubated for 24 h with 5% CO_2. Selection with 200 μg/mL hygromycin B (Calbiochem) was initiated; after 2 weeks, independent clonal foci were harvested and assayed for cGI PDE activity.

Of 50 clonal foci isolated after RcGIP1 transfection, 12 exhibited cAMP PDE activity (0.65−2.8 nmol of cAMP hydrolyzed/min/mg of protein) up to 200-fold higher than that in untransfected cells or cells transfected with pBPV alone (10−20 pmol of cAMP/min/mg of protein). Based on specific activity of purified rat adipocyte cGI PDE (Degerman et al., 1987), ∼5 μg of cGI PDE can accumulate in a single dish (10 cm^2) in some cultures, as much as might be in adipocytes from ∼200 rats. Of 26 clonal foci isolated after HcGIP2 transfection, seven exhibited specific activity between 0.4 and 0.8 nmol of cAMP/min/mg of protein. Activities of positive clones were inhibited (at least ∼75%) by 0.5 μM cilostamide (OPC 3689), a specific cGI PDE inhibitor.

Expression of HcGIP2 in Baculovirus-Infected Sf9 Cell

Construction of Vectors. A partial HcGIP2 cDNA (n.2) encoding a truncated ∼54 kDa cGI PDE (amino acids 613−1108), which included the conserved catalytic domain, was amplified by PCR (GeneAmp PCR system 9600, Perkin Elmer). The ∼4 kb *Eco*R1 restriction fragment containing the entire open reading frame (full-length) of HcGIP2 (Meacci et al., 1992) was used as template with 100 ng of sense primer, 5′-CTG GTT GCG AGAATTC ATG CGA ACA GAT GAC ACT GC [corresponding to nt 1840−1856 plus an *Eco*R1 site and ATG initation codon] and antisense primer, 5′-CTC GCT CCG GCG AGAATTC TTA CTG AGG GGT CTG GTC CA [corresponding to nt 3311−3328 plus an *Eco*R1 site and stop codon] and Taq polymerase with denaturation (94 °C for 1 min), annealing at 55 °C for 1 min, extension at 72 °C for 2 min for 30 cycles, and final extension at 72 °C for 10 min. The PCR product (n.2) was purified by electrophoresis (1% agarose) and Gene Clean II (Bio 101) and restricted with *Eco*R1. Both n.2 and the ∼4 kb HcGIP2 cDNA fragments were ligated into *Eco*R1- and alkaline phosphatase-digested Baculovirus transfer vector pVL1393 (Pharmingen). The resulting plasmids pVL/n.2 and pVL/4 kb were used to transform competent DH5α.

A second truncated HcGIP2, approximately 100 amino acids longer than n.2 and encoding a protein of ∼69 kDa, was constructed from the deletion mutant AcGIΔ5 (Δ5), designed by Pellai et al. (1994) to encode the initiation ATG and amino acids 511−1141 of HcGIP2. This deletion mutant was supplied as a yeast expression vector, pAcGIΔ5 (Dr. J. Colicelli, UCLA). The plasmid was purified and digested with *Nco*1 and *Not*1; the [Δ5 Nco−*Not*1] HcGIP2 fragment was cloned into pBluescript (KS) HcGIP2 that had been digested with *Nco*1−*Not*1. The Δ5 HcGIP2 deletion was removed by digestion with *Eco*R1 and cloned into pVL1393 (pVL/Δ5). Sequence analysis confirmed the 5′ *Eco*R1−*Nco*1 (ATG) sequence as well as ∼300 bp downstream [since in Δ5 only the 5′ portion was generated by PCR (Pellai et al., 1994), we did not verify other downstream sequences]. pVL/Δ5 and a second full length construct pVL/4 kb-2 were transfected into different Sf9 cells than used in studies with pVL/4 kb and pVL/n.2. This may account for the observed differences in cGI PDE activities.

Production of Recombinant Baculovirus and Transfection. Transfer of the full-length (pVL/4 kb and pVL/4 kb-2) and truncated (pVL/n.2 and pVL/Δ5) HcGIP2 cDNAs to the AcNPV (*Autographa california* nuclear polyhedrosis virus) genome was achieved by homologous recombination with the resident polyhedrin gene after calcium phosphate cotransfection of recombinant pVL1393 plasmids (2 μg) into Sf9 insect cells (3×10^6 cells/25 cm^2 tissue culture flask) with wild-type, linearized AcNPV DNA (0.5 μg) using the

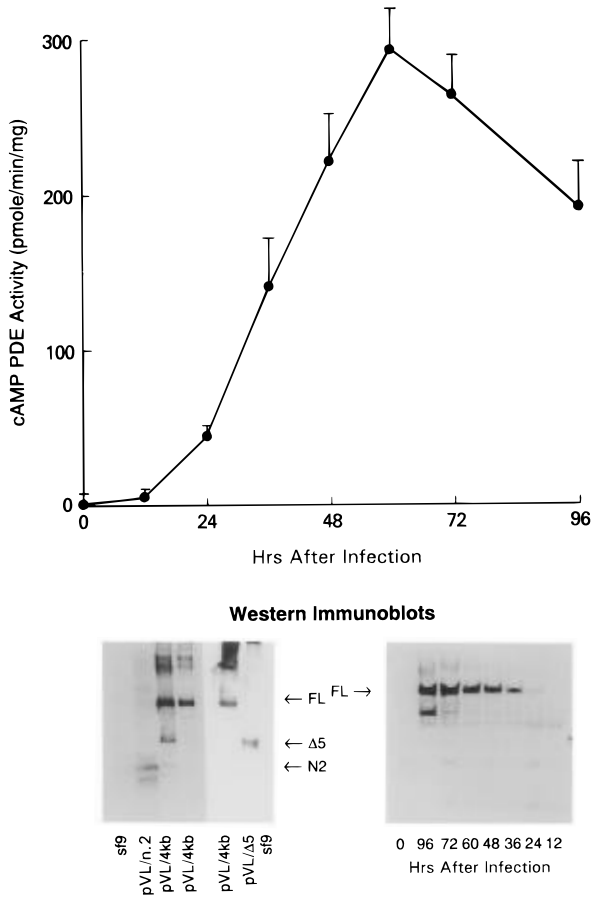

FIGURE 1: Time course of expression of recombinant HcGIP2 in Sf9 cells. Sf9 cells ($\sim 10^7$ cells per 75 cm^2 tissue culture flask) infected with full-length pVL/4 kb recombinant virus (●) were incubated for the indicated times, harvested, and centrifuged (1000*g*, 10 min); cell pellets were suspended in buffer A (Table 4), sonified, and assayed for cAMP PDE activity (upper panel) and SDS−PAGE/Western blotting (lower right panel) as described in Materials and Methods. Values for PDE activity represent mean ± SEM ($n = 5$ experiments). (lower left panel) Three different experiments in which lysates of uninfected Sf9 cells and Sf9 cells infected for 60 h with full-length pVL/4 kb and pVL/4 kb-2 and truncated pVL/n.2 and pVL/Δ5 were subjected to SDS−PAGE and Western immunoblots. In one experiment, there was apparently significant proteolysis of the full-length pVL/4 kb, yielding an immunoreactive band with M_r similar to pVL/Δ5.

"Baculogold Transfection Kit" (PharMingen). Transfected Sf9 cells (CRL 1711, American Type Culture Collection) [maintained at 27 °C in serum-free insect culture medium (Gibco Labs) supplemented with 10% heat-inactivated bovine fetal serum] were incubated in 3 mL of culture medium for 4 days at 27 °C, when supernatant containing recombinant virus was collected and used to infect fresh Sf9 cells. Amplification was repeated 3−4 times; the amplified recombinant virus was stored at 4 °C and protected from light.

As seen in Figure 1, an increase in PDE activity and immunoreactive material (Figure 1, lower panel) was observed within 24 h of infecting Sf9 cells with full-length pVL/4 kb, with maximal activity at ~60 h postinfection. Sf9 cells were, therefore, routinely infected [1 mL of virus-containing supernatant, 10^7 cells/75 cm^2 tissue culture flask (Corning, Inc.)] and harvested at ~60 h after infection (Figure 1, lower panel).

Antibody Production and Western Blot Analysis of Recombinant RcGIP1 and HcGIP2 Expressed in NIH 3006 Fibroblasts and Sf9 Cells

Based on the deduced sequence of RcGIP1 (Taira et al., 1993), RcGIP1 peptide NT [amino acids (aa) 2−16, in the N-terminal region], RcGIP1 peptide CD [aa 736−749 in the catalytic domain, which primarily contains amino acids in the 44 amino acid insertion in the catalytic domain of RcGIP1 (Taira et al., 1993)], and RcGIP1 peptide CT (aa 1078−1093, in the C-terminal region) were used to immunize rabbits after conjugation of peptides (synthesized by Dr. R. Schackmann, University of Utah) to keyhole lympet hemocyanin (CD peptide) or ovalbumin (NT and CT peptides) using Imject activated immunogen conjugation kits and Ellman's reagent [5′,3′-dithiobis(2-nitrobenzoic acid) according to the manufacturer's directions (Pierce, USA)].

IgG fractions, purified by chromatography on Protein G Sepharose 4 Fast Flow (Pharmacia) after passage of serum over columns of immobilized *Escherichia coli* lysate (5′,3′ Inc.), were stored at −20 °C in 50% glycerol. Rabbit anti-human platelet cGI PDE antibodies were affinity-purified by a modification of the method of Olmstead (Murashima et al., 1990; Tanaka et al., 1991); rabbit anti-RcGIP1 peptide RD (aa 423−440 in the regulatory domain of RcGIP1) antibodies were purified as described (Degerman et al., 1994; Rasçon et al., 1994).

Lysates from NIH 3006 or Sf9 cells were subjected to SDS−PAGE (8% or 10% gel, 90−120 min, 100 V). Proteins were stained with Coomassie Blue or transferred to nitrocellulose membranes (0.45 μm). After blotting, membranes were incubated overnight in blocking solution [1% I-Block (Amersham)/0.3% Tween 20 in PBS or BSA in 20 mM Tris-HCl, pH 7.5/500 mM NaCl], washed three times for 10 min in PBS/0.05% Tween 20, and incubated for 1−2 h at room temperature with anti-RcGIP1 peptide or anti-platelet cGI PDE antibodies. Membranes were washed three times with PBS/0.05% Tween 20 for 10 min; bound antibodies were detected with anti-rabbit IgG-linked alkaline phosphatase (Promega) or with anti-rabbit IgG linked to horseradish peroxidase (1 h, room temperature) and revealed by chemiluminescence (Amersham).

Protein content was determined by the Bio-Rad protein assay kit or, especially for experiments in which detergents were present, the Bicinchoninic Acid Protein Assay Reagent Kit (Pierce). Protein concentrations of the HPLC fractions were determined using ISS Protein-Gold Protein Assay Reagent (Integrated Separation Systems). Bovine serum albumin was used as standard in all protein assays.

Preparation of Tryptic Phosphopeptides from Authentic Rat Adipocyte cGI PDE and Recombinant RcGIP1 Phosphorylated in Vitro by cAMP-PrK

Solubilized adipocyte cGI PDE (600 μL packed cell volume, 200 pmol/min) was ^{32}P-phosphorylated by cAMP-PrK and immunoisolated as described by Rasçon et al. (1994). Fibroblast RcGIP1 was solubilized with $C_{13}E_{12}$ and NaBr as described in the legend to Table 5 and phosphorylated by cAMP-PrK (100 units/mL, Sigma) in the presence of 50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 40 μM [γ-^{32}P]-ATP, 1 mM DTT, 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 1 μg/mL antipain for 30 min at 37 °C. ^{32}P-phosphorylated immunoisolated adipocyte cGI PDE and fibroblast

Table 1: cAMP Hydrolysis by Recombinant RcGIP1 and HcGIP2 Isoforms[a]

NIH fibroblasts clone	apparent K_m (μM)	Sf9 cells	apparent K_m (μM)
RcGIP1 28	0.21 ± 0.1	full length HcGIP2 pVL/4 kb[b]	0.15 ± 0.03
RcGIP1 39	0.22 ± 0.0	pVL/4 kb-2	0.12 ± 0.01
HcGIP2 2	0.25 ± 0.0	truncated HcGIP2 pVL/n.2[b]	0.22 ± 0.06
HcGIP2 3	0.24 ± 0.1	pVL/Δ5	0.12 ± 0.03

[a] Confluent fibroblasts (100 mm dishes) were washed rapidly with cold PBS, harvested with a rubber policeman, suspended, and sonified (on ice twice for 30 s using a Sonic Dismembrator 50 (Fisher Scientific) at 20−30% output power) in 1 mL of cold homogenization buffer (100 mM TES, pH 7.4/5 mM $MgSO_4$/1 mM EDTA/100 μM EGTA/1 mM benzamidine/5 μg/mL each of leupeptin, pepstatin A, aprotinin/0.5 mM Pefabloc/10% glycerol). Sf9 cells were collected by centrifugation (1000*g*, 10 min), suspended in 1 mL of buffer A (50 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM EDTA, 0.1 mM EGTA, 3 mM benzamidine, 10 μg/mL each of pepstatin and leupeptin and 0.5 mM Pefabloc) containing 10% glycerol and sonicated. PDE activities in whole lysates were assayed with 0.1−10 μM [^{3}H]cAMP. K_m values were obtained by linear regression analysis (Stat View) of Lineweaver−Burk plots. Values represent mean ± 1/2 range (lysates from two cultures each assayed in duplicate). [b] For these two recombinants K_m values were determined by weighted nonlinear least-squares regression analysis [Gauss-Newton method (Cleland, 1979)]. In these experiments a K_m of 0.14 was observed with a human myocardial microsomal cGI PDE preparation.

RcGIP1 were subjected to SDS−PAGE, blotted onto nitrocellulose membranes, and detected by exposure on a Fuji phosphoimager. Membrane-bound ^{32}P-cGI PDEs were excised and digested with trypsin essentially as described by Aebersold et al. (1987). Pieces containing ^{32}P-cGI PDE were incubated for 30 min at 37 °C in 0.6% acetic acid and 0.5% polyvinylpyrrolidone, washed three times with distilled water and incubated with trypsin (each with 1 μg in 200 μL of 50 mM ammonium bicarbonate) for 16 h at 37 °C. The eluate from the filter (>90% of radioactivity) was vacuum centrifuged, and the dried peptides were oxidized with formic acid (final volume 50 μL) for 60 min at 4 °C. Oxidized peptides were diluted in 450 μL of water, subjected to vacuum centrifugation, dissolved in 50 mM ammonium bicarbonate (50 μL), and incubated with trypsin for 16 h before addition of 140 μL of formic acid/acetic acid/H_2O (44:156:1800 v/v/v) buffer, pH 1.9. After centrifugation (10000*g*, 5 min), 180 μL was subjected to vacuum centrifugation. (20 μL was left to avoid touching the pellet with the pipet tip). Finally, the peptides were dissolved in 200 μL of SDS-sample buffer and subjected to Tricine SDS/urea−PAGE (Schagger et al., 1987). Gels were analyzed with the Fuji Base 2000 phosphoimager.

Assay of cAMP Phosphodiesterase Activity in NIH 3006 Fibroblasts and Sf9 Cells

For cAMP PDE assays (Kincaid & Manganiello, 1988), samples were incubated at 30 °C in a total volume of 0.3 mL containing 50 mM HEPES, pH 7.4/0.1 mM EDTA/8.3 mM $MgCl_2$/0.1 μM [^{3}H]cAMP (18 000 cpm). Activity was proportional to enzyme concentration, and substrate hydrolysis was less than 20%. cGI PDE activity was measured as the cAMP PDE activity inhibited by 0.5 μM cilostamide (OPC 3689) or OPC 3911, specific cGI PDE inhibitors (Manganiello et al., 1990; Beavo & Reifsnyder, 1990; Thompson, 1991; Nicholson et al., 1991).

RESULTS AND DISCUSSION

Characterization of Recombinant cGI PDEs Expressed in NIH 3006 Fibroblasts and Sf9 Cells: Substrate Affinities and Inhibitor Sensitivities. K_m values for full-length RcGIP1 and HcGIP2 recombinants from fibroblasts were similar to those for cGI PDE from adipocytes, human myocardial microsomes, and other tissues (Manganiello et al., 1990) (Table 1). RcGIP1 and HcGIP2 recombinants were inhibited by cGMP, cilostamide, and vesnarinone [a drug in clinical trials for treatment of congestive heart failure (Rapundalo et al., 1988; Feldman et al., 1993)] with IC_{50} values similar to those for cGI PDEs from rat adipose tissue, human and bovine heart, and human kidney (Harrison et al., 1986; Degerman et al., 1987; Movsesian et al., 1991; Matsuoka et al., 1993) (Table 2). HcGIP2 was more sensitive than RcGIP1 to inhibition by cGMP (Table 2), as previously reported for cGI PDEs purified from bovine myocardium and rat adipose tissue (Harrison et al., 1986; Degerman et al., 1987).

Table 2: Inhibition of Recombinant RcGIP1 and HcGIP2 Expressed in NIH 3006 Fibroblasts[a]

	clone	IC_{50} cGMP (nM)	OPC 3689 (nM)	vesnarinone (μM)	rolipram (μM)
RcGIP1	28	235 ± 7	40 ± 1	9.5 ± 3.5	>100
	39	225 ± 24	41 ± 2	8.3 ± 0.5	>100
HcGIP2	2	44 ± 12	13 ± 3	4.9 ± 2.1	>100
	3	54 ± 5	25 ± 8	8.4 ± 1.8	>100

[a] cAMP PDE activities in fibroblast lysates prepared as described in Table 1 were assayed with 0.1 μM [^{3}H]cAMP without or with six concentrations of the indicated inhibitors. Activity in the absence of inhibitor was taken as 100%, and IC_{50} values were estimated from graphical analyses of inhibition curves. Values represent mean ± 1/2 range (lysates from two culture dishes assayed in duplicate).

The deduced sequences of cGIP1 and cGIP2 isoforms are very similar in their C-terminal catalytic domains but quite divergent in their N-terminal portions (Meacci et al., 1992; Taira et al., 1993). It has been suggested that removal of the N-terminal portion influences the catalytic properties of the C-terminal catalytic domain (Kasuya et al., 1995), but, as shown in Table 1, two truncated HcGIP2 recombinants from Sf9 cells (pVLn.2 and pVLΔ5), which lack most of the N-terminal region, did not exhibit significantly higher K_m values than full length HcGIP2 (pVL4kb and pVL4kb-2). Similar results were observed with other truncated RcGIP1 and HcGIP2 recombinants (data not shown). Truncated and full-length HcGIP2 recombinants from Sf9 cells and a human myocardial microsomal cGI PDE preparation were inhibited by OPC 3911 with similar IC_{50} values (20−50 nM) (data not shown). These results suggest that the considerable differences in deduced sequences of the N-terminal regulatory domains are not reflected in marked differences in substrate affinities or inhibitor sensitivities. HcGIP2 was, however, more sensitive than RcGIP1 to inhibition by cGMP (Table 2). Despite the overall similarity in deduced sequences of RcGIP1 and HcGIP2 catalytic domains, the sequences of these two cGI PDEs differ in the 44 amino acid insertion that is unique to cGI PDE catalytic domains. This insertion may not only distinguish cGI PDE

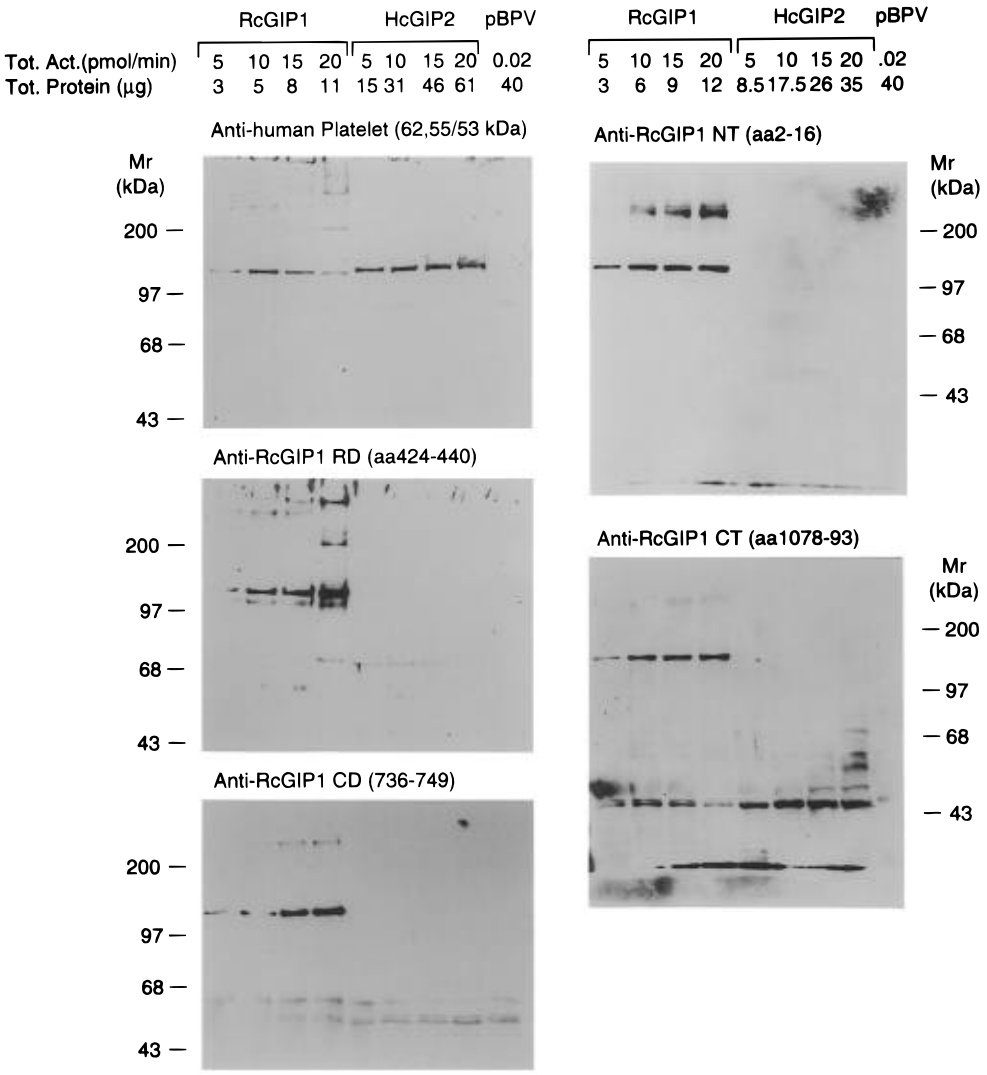

FIGURE 2: Western immunoblots of recombinant RcGIP1 and HcGIP2 expressed in NIH 3006 cells. Portions of whole lysates containing the indicated amount of total protein and PDE activity from cells transfected with pBPV alone or recombinant plasmids were subjected to SDS−PAGE (8% gel) and Western immunoblots with indicated anti-RcGIP1 peptide and anti-platelet cGI PDE antibodies. Anti-RcGIP1 peptide antibodies cross-reacted with authentic adipocyte cGI PDE (data not shown). Nonspecific background was more pronounced with adipocyte than with RcGIP1 recombinant material, except for anti-RD (Rascon et al., 1994).

catalytic domains from those of other PDE families and identify catalytic domains of different cGI PDE subfamilies (Meacci et al., 1992; Taira et al., 1993) but may also determine sensitivity of cGI PDEs to cGMP.

Immunological Reactivities. Full-length RcGIP1 and HcGIP2 exhibited the expected subunit molecular mass of cGI PDEs (Figure 2). The small difference in their apparent sizes corresponds to the molecular mass values predicted from their deduced amino acid sequences, ~123 kDa for RcGIP1 and ~125 kDa for HcGIP2. The anti-human platelet cGI PDE antibody recognizes both fibroblast RcGIP1 and HcGIP2. This is not surprising, since anti-platelet cGI PDE antibodies were raised against catalytically active C-terminal fragments (62, and 55/53 kDa) of the purified platelet cGI PDE (Degerman et al., 1994), and since the deduced sequences of cGIP1 and cGIP2 catalytic domains are quite similar to each other except for the 44 amino acid insertion.

As also seen in Figure 2, RcGIP1 cross-reacted with four different anti peptide antibodies directed against peptide sequences from the regulatory domain (RD), the additional region of the catalytic domain (CD), N-terminal region (NT), and C-terminal region (CT) of RcGIP1. Those sequences are specific to RcGIP1 and are not found in HcGIP2 (Meacci et al., 1992; Taira et al., 1993). The anti-RcGIP1 peptide antibodies were selective since, with the indicated amounts of protein and PDE activity, little or no cross-reactivity was observed with HcGIP2. Material from control cells (pBPV alone) did not cross-react with antiplatelet or antipeptide antibodies. The anti-RcGIP1 peptide antibodies also cross-reacted with authentic adipocyte cGI PDE on Western blots (Rasçon et al., 1994; data not shown).

In solubilized authentic adipocyte cGI PDE, the major site phosphorylated by cAMP-PrK *in vitro* (Rasçon et al., 1994) is Ser 427, whereas Ser 302 is the major site phosphorylated in intact cells treated with insulin or agents that increase cAMP (Rahn et al., 1996). Solubilized fibroblast RcGIP1 and authentic adipocyte cGI PDE were phosphorylated by cAMP-PrK *in vitro* and immunoprecipitated by anti-RcGIP1 peptide antibodies (Figure 3). Anti-CD and anti-CT were less efficient than anti-RD and anti-NT. Trypsin digestion of phosphorylated RcGIP1 and authentic adipocyte cGI PDE yielded [^{32}P]peptides with identical electrophoretic mobilities in Tricine-urea/SDS−PAGE (Figure 3, insert), suggesting that the same peptides were phosphorylated *in vitro* in

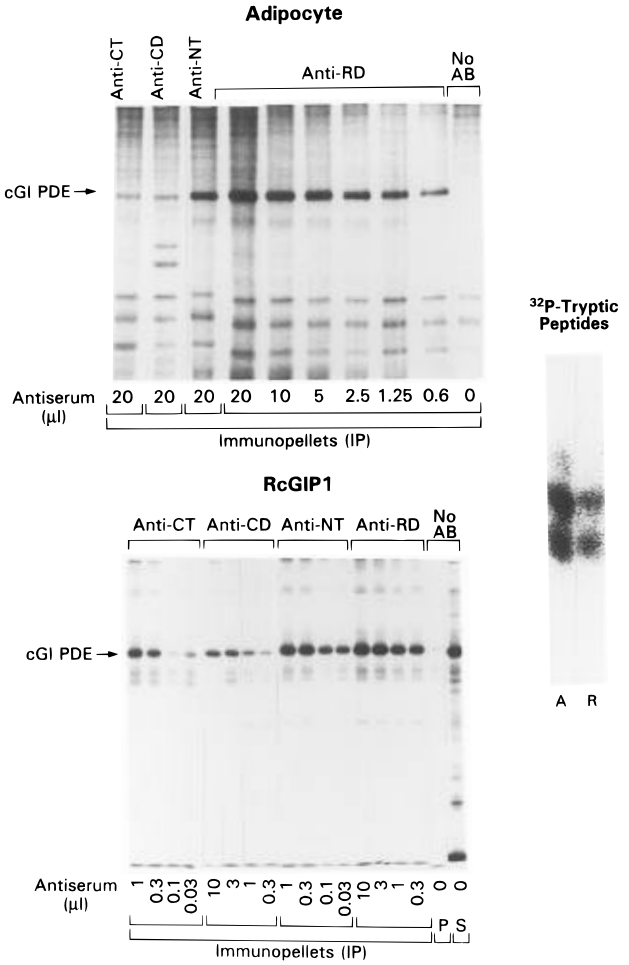

FIGURE 3: Immunoprecipitation of authentic adipocyte cGI PDE and recombinant RcGIP1 phosphorylated *in vitro* by cAMP-PrK. Samples of solubilized adipocyte particulate fractions (upper panel) or recombinant particulate RcGIP1 (lower panel) (each containing ~30 pmol/min cGI PDE activity) were phosphorylated with [γ-^{32}P]-ATP and cAMP-PrK and incubated (16 h, 4 °C) with antipeptide antibodies and then for 15 min with *Staphylococcus aureus* protein A as previously described (Degerman et al., 1994; Rascon et al., 1994). Immunoprecipitates were subjected to SDS−PAGE (7% gels), and dried gels were analyzed using the Fuji Base 2000 phosphoimager. In the lower panel, ^{32}P-RcGIP1 was also incubated without antibody and centrifuged to yield pellet (P) and supernatant (S) fractions. (Inser) Tricine SDS/urea−PAGE of tryptic [^{32}P]-peptides. Authentic ^{32}P-adipocyte cGI PDE was immunoprecipitated, subjected to SDS−PAGE, electroblotted to a nitrocellulose membrane, excised, and digested with trypsin. ^{32}P-RcGIP1 was treated identically but was not immunoprecipitated before SDS−PAGE. ^{32}P-Tryptic peptides were prepared from native adipocyte cGI PDE (A) and recombinant fibroblast RcGIP1 (R) and separated by Tricine SDS/urea−PAGE as described in Materials and Methods.

solubilized RcGIP1 and adipocyte cGI PDE. It is hoped that expression of recombinant RcGIP1 in insulin-responsive cells will allow dissection of pathways involved in regulation of cGI PDEs and identification by direct sequencing of the *in vivo* phosphorylation site(s) for cAMP-PrK and an insulin-sensitive kinase thought to be responsible for hormonal regulation of cGI PDE.

Subcellular Distribution. The deduced sequences of both RcGIP1 and HcGIP2 (Meacci et al., 1992; Taira et al., 1993) predict hydrophobic N-terminal regions containing several transmembrane helical segments. After centrifugation (105000g, 45 min) of homogenates, full-length HcGIP2 and RcGIP1 from fibroblasts (Table 3) and full-length HcGIP2 from Sf9 cells (Table 4) were found predominantly in

Table 3: Subcellular Distribution of Recombinant RcGIP1 and HcGIP2 Activities in NIH 3006 Fibroblasts[a]

	RcGIP1			
	clone 28		clone 39	
cell fraction	PDE activity (nmol/min)	protein (mg)	PDE activity (nmol/min)	protein (mg)
whole lysate	9.1 ± 1.9	1.6 ± 0.2	3.4 ± 0.6	1.8 ± 0.2
cytosol	1.2 ± 0.1	0.8 ± 0.0	0.3 ± 0.1	0.9 ± 0.2
particulate fraction (100000*g*, 45 min)	12.2 ± 2.6	0.6 ± 0.1	3.4 ± 0.5	0.7 ± 0.7
	n = 2		*n* = 4	
	HcGIP2			
	clone 2		clone 3	
cell fraction	PDE activity (nmol/min)	protein (mg)	PDE activity (nmol/min)	protein (mg)
whole lysate	0.4 ± 0.1	0.9 ± 0.1	0.4 ± 0.1	0.8 ± 0.0
cytosol	0.08 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.3 ± 0.0
particulate fraction	0.34 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.2 ± 0.0
	n = 2		*n* = 2	

[a] Confluent fibroblasts were harvested and sonified in 1 mL of homogenization buffer as described in Table 1. A portion of the whole lysate (0.2 mL) was kept on ice; 1.2 mL of homogenization buffer was added to the remainder, and the mixture was centrifuged (100000*g*, 45 min). Samples of cytosol and particulate fractions (suspended in 1 mL of homogenization buffer and sonicated on ice for 30 s) were assayed for protein content and cAMP PDE activity (0.1 μM [^{3}H]cAMP). Values for total PDE activity or protein content of the cell lysates represent the mean ± $^1/_2$ range or SD of two or four experiments.

particulate fractions. On the other hand, in Sf9 cells almost all activity of the truncated HcGIP2 forms, pVL/n.2 and pVL/Δ5, encoding amino acids 613−1108 and 511−1141, respectively, was cytoplasmic (Table 4). These results strongly suggest that determinants for cGI PDE association with intracellular membranes may be located in this hydrophobic region, perhaps within the predicted transmembrane segments.

Platelet cGI PDE, for example, is thought to be predominantly cytosolic (Grant & Colman, 1984; MacPhee et al., 1986; Alvarez et al., 1986; Degerman et al., 1994), whereas adipocyte and hepatocyte isoforms are predominantly particulate (Kono et al., 1975; Degerman et al., 1987; Pyne et al., 1987; Boyes & Loten, 1988, 1989). Myocardial cGI PDE activity has been found in cytosol as well as associated with sarcoplasmic reticulum (SR) (Harrison et al., 1986; Kauffman et al., 1987; Kithas et al., 1989; Silver et al., 1990; Movsesian et al., 1991; Weishaar et al., 1992; Smith et al., 1993; Lugnier et al., 1993). It is possible that particulate and cytosolic cGI PDEs serve different functions, the SR isoform being closely coupled to regulation of cAMP-PrK-catalyzed phosphorylation of phospholamban and Ca^{2+}-uptake into the SR. An ~73 kDa hepatocyte cGI PDE was purified after being released from particulate fractions by treatment with chymotrypsin (Boyes & Loten, 1988). A placental cDNA encoding a presumed transcriptional variant of HcGIP2 with a predicted MW of ~74 000 (similar to pVLΔ5) was expressed in Sf9 cells and also found primarily in the cytosolic fraction (Kasuya et al., 1995). Whether removal of the membrane-association domains by proteolysis, alternative transcription, or mRNA splicing accounts for generation of some cytosolic cGI PDE isoforms (Smith et al., 1993; Kasuya et al., 1995) remains to be proven.

Solubilization and Partial Purification. As seen in Table 5, full-length RcGIP1 from fibroblasts was solubilized by sonication in buffer containing 1% $C_{13}E_{12}$, the nonionic

Table 4: Subcellular Distribution of Recombinant HcGIP2 in Sf9 Cells[a]

	total PDE activity (nmol/min)				
	full length			truncated	
cell fraction	pVL/4 kb[*]	pVL/4 kb-1[**]	pVL/4 kb-2[***]	pVL/n.2[****]	pVL/Δ5[***]
whole lysate	1.2 ± 0.1	2.0 ± 0.2	3.4 ± 0.0	1.0 ± 0.1	4.7 ± 0.4
cytosol	0.1 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.9 ± 0.1	4.1 ± 0.6
particulate fraction	1.0 ± 0.1	1.4 ± 0.2	2.8 ± 0.1	0.03 ± 0.0	0.5 ± 0.1

[a] Mean ± SEM, [*]$n = 7$, [**]$n = 4$, [****]$n = 6$; mean ± 1/2 range, $n = 2$[***]; p.VL/4 kb, recombinant virus not purified; pVL/4 kb-1, recombinant virus purified from stock pVL/4 kb by plaque assay (Summers & Smith, 1987); [***]pVL/4 kb-2 and pVL/Δ5, recombinant viruses not purified. After infection (10^7 Sf9 cells, 1 mL of virus, 60 h, 27 °C), cells were collected by centrifugation (1000 g, 10 min), suspended in 1 mL of buffer A containing 40% glycerol, and sonicated [three times, 20 s each on ice with model W-225 (Heat Systems-Ultrasonics, Inc.) at 30% Duty Cycle and Output Control 3]. Lysates were diluted 10-fold with buffer A without glycerol and centrifuged (100000*g*, 45 min) to generate cytosolic and particulate fractions. For pVL/4 kb-2 and pVL/Δ5, cells were sonified in buffer A with 10% glycerol and not diluted before centrifugation.

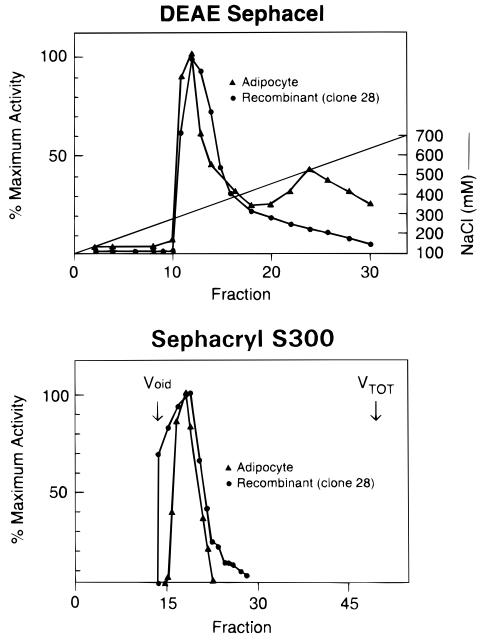

FIGURE 4: Chromatography of solubilized rat adipocyte particulate and recombinant RcGIP1 fractions on DEAE Sephacel and Sephacryl S-300. (Top) Solubilized adipocyte cGI PDE (▲) (600 pmol/min) and solubilized recombinant RcGIP1 (●) (800 pmol/min) were applied to a DEAE Sephacel column (2 mL) equilibrated in 50 mM Tris, pH 7.4/5 mM $MgCl_2$/1 mM EDTA/0.03% $C_{13}E_{12}$/5% glycerol/100 mM NaCl and eluted (0.3 mL/min) in the same buffer with a linear gradient of 100−700 mM NaCl. cGI PDE activity in eluted fractions (1 mL) is presented as percent of maximal activity eluted in the peak fraction (25 and 19 pmol/min/mL for adipocyte cGI PDE and recombinant RcGIP1, respectively, in fraction 12). (Bottom) Solubilized adipocyte cGI PDE (200 pmol/min) and solubilized recombinant RcGIP1 (●) (200 pmol/min) were applied to a column (40 cm × 0.9 mm i.d.) of Sephacryl S-300 equilibrated and eluted (3 mL/h) in 50 mM Tris, pH 7.4/5 mM $MgCl_2$/1 mM EDTA/0.03% $C_{13}E_{12}$/100 mM NaCl. cGI PDE activity in eluted fractions (0.5 mL) is expressed as percent of maximal activity recovered in the peak fraction (∼20 pmol/min/mL for rat adipocyte cGI PDE and recombinant RcGIP1 in fraction 19).

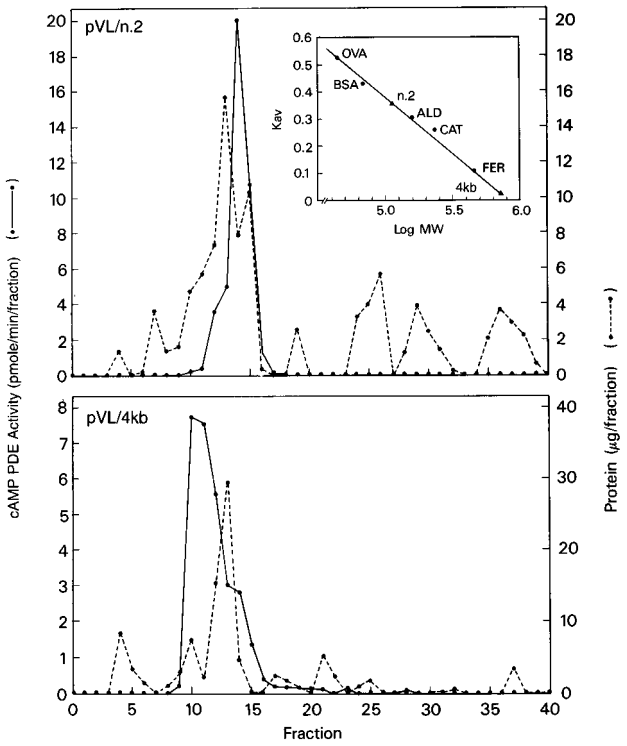

FIGURE 5: HPLC gel-filtration chromatography of HcGIP2 recombinants. After partial purification by chromatography on DEAE-Sephacel, truncated cytosolic pVL/n.2 and solubilized full-length particulate pVL/4 kb were subjected to size-exclusion chromatography on a TSK-G3000SW HPLC column (60 cm × 7.5 mm i.d.) equilibrated and eluted (1 mL/min, 35 bar pressure) with buffer A (Table 4) without glycerol containing 0.16% $C_{13}E_{12}$ and 250 mM NaBr. Fractions (1 mL) were collected into test tubes containing 1 mL of ice-cold buffer A (40% glycerol) to preserve enzyme activity, and portions were assayed for protein and cAMP PDE activity. (Inset) Apparent molecular sizes of the recombinant HcGIP2 PDEs (▲) were determined by comparison with standards (●) (Pierce): FER, ferritin (M_r 440K); CAT, catalase (M_r 240K); ALD, aldolase (M_r 158K); BSA, bovine serum albumin (M_r 67K); OVA, ovalbumin (M_r 43K). The void volume of the column was determined with Blue Dextran [Gel-filtration Calibration Kit (Pharmacia)]. The same elution profile was observed if dilute or concentrated (Centricon) samples were applied.

detergent used to solubilize particulate adipocyte cGI PDE (Degerman et al., 1987). Since direct sonication of isolated adipocytes is impractical due to the abundance of triglycerides, to compare properties of RcGIP1 and authentic adipocyte cGI PDE, isolated particulate fractions, rather than intact cells, were solubilized in the presence of salt and detergent, alone and in combination. As seen in Table 6, RcGIP1 was somewhat more difficult to solubilize than adipocyte cGI PDE, indicating that there may be subtle differences between fibroblast and adipocyte membranes and/or the manner in which RcGIP1 and adipocyte cGI PDE are inserted into intracellular membranes. It is also possible that, in fibroblasts, "overexpression" of RcGIP1 may result in formation of insoluble aggregates that resist disruption.

As seen in Figure 4 (upper panel), after solubilization in the presence of $C_{13}E_{12}$, both RcGIP1 and authentic adipocyte cGI PDE eluted from DEAE Sephacel at ∼300 mM NaCl. RcGIP1 activity eluted in a somewhat broader peak, but a greater proportion of the total adipocyte activity eluted at higher (>500 mM) NaCl. Both RcGIP1 and adipocyte cGI

Table 5: Solubilization of Recombinant RcGIP1 Activity in NIH 3006 Fibroblasts[a]

		clone			
		RcGIP1 28		RcGIP1 39	
treatment	cell fraction	PDE activity (nmol/min)	protein (mg)	PDE activity (nmol/min)	protein (mg)
$C_{13}E_{12}$ (1%)	whole lysate	16.6 ± 3.7	1.2 ± 0.3	4.3 ± 0.2	1.0 ± 0.1
	solubilized	14.1 ± 3.4	0.9 ± 0.2	5.2 ± 0.9	0.8 ± 0.2
	particulate fraction	5.8 ± 1.2	0.4 ± 0.1	0.8 ± 0.1	0.4 ± 0.3
NaBr (0.5 M)	whole lysate	12.9 ± 1.0	1.1 ± 0.2	4.5 ± 0.3	1.0 ± 0.1
	solubilized	2.6 ± 0.3	0.9 ± 0.3	1.0 ± 0.2	0.8 ± 0.0
	particulate fraction	12.0 ± 2.5	0.3 ± 0.2	3.4 ± 0.7	0.2 ± 0.0

[a] Confluent fibroblasts were harvested as described in Table 1 and sonicated (3 times, 20 s each, on ice in a Sonifier Cell Disruptor 350, output control 2, 50% duty cycle) in homogenization buffer (2 mL) containing 1% $C_{13}E_{12}$ (a nonionic polyoxyethylene detergent) or 0.5 M NaBr. As shown in Table 3, in cells homogenized in the absence of salt and detergent, >80% of RcGIP1 and HcGIP2 activities were recovered in the particulate fraction. A portion (0.3 mL) was kept on ice for assay of protein and PDE activity as described in Materials and Methods; the remainder (1.7 mL) was centrifuged for 45 min at 105000*g* to yield supernatant (solubilized particulate cGI PDE) and particulate fractions (which were suspended in 1 mL of homogenization buffer). Values for total PDE activity and protein content represent mean ± SD of three determinations (lysates from three dishes assayed in duplicate). Similar effects of $C_{13}E_{12}$ on solubilization of HcGIP2 clone 6 and clone 4 were observed (data not shown).

Table 6: Solubilization of Particulate Recombinant RcGIP1 and Authentic Adipocyte cGI PDEs[a]

	percentage of total cGI PDE activity in supernatant	
	adipocyte	RcGIP1 (clone 28)
buffer	4.4 ± 5.2*	3.8 ± 3.3*
$C_{13}E_{12}$ (1%)	38 ± 16	23 ± 14
NaBr (500 mM)	16 ± 12	10 ± 5
$C_{13}E_{12}$ plus NaBr	89 ± 23	64 ± 7

[a] Adipocytes were prepared from rat (Sprague-Dawley, 36 days old) epididymal fat pads as previously described (Degerman et al., 1987). Cells (10% suspension) were homogenized at room temperature in 100 mM TES, pH 7.4/5 mM $MgSO_4$/1 mM EDTA/100 μM EGTA/1 mM benzamidine/5 μg/mL each of leupeptin, pepstatin A, aprotinin/10% glycerol, and immediately cooled to 4 °C. Confluent fibroblasts were washed rapidly with cold PBS, harvested with a rubber policeman, suspended in homogenization buffer, and sonicated (30 s three times on ice). Fat cell and fibroblast homogenates were centrifuged (100000*g*, 60 min) to yield particulate and supernatant fractions. Particulate fractions from fibroblasts (one dish) or adipocytes (200 μL of packed cell volume) were homogenized in 1 mL of buffer containing 500 mM NaBr, 1% $C_{13}E_{12}$, or both, incubated (1 h, on ice), and centrifuged (100000*g*, 60 min) to yield particulate and supernatant fractions, which were assayed for PDE activity as described in Materials and Methods. Values represent mean ± SD, *$n = 5$ preparations (adipocytes) and $n = 3$ preparations (RcGIP1).

PDE eluted at or close to the void volume of Sephacryl S-300, indicating the presence of multimers or large aggregates (Figure 4, lower panel) as reported with material solubilized from freshly prepared adipocytes (Makino & Kono, 1980). Although we and Makino and Kono (1980) did not correct for detergent binding, the apparent size of RcGIP1 and authentic adipocyte cGI PDEs is larger than that previously reported for a proteolyzed, partially purified adipose tissue cGI PDE (Degerman et al., 1987).

Both solubilized full-length pVL/4 kb and truncated cytosolic pVL/n.2 HcGIP2 from Sf9 cells were partially purified by chromatography on DEAE Sephacel (data not shown); material eluted at 300 mM NaBr was subjected to HPLC size-exclusion chromatography (Figure 5). As observed with RcGIP1 from fibroblasts, solubilized full-length HcGIP2 eluted as multimers or large aggregates (> 400 kDa); truncated pVL/n.2 eluted as a dimer (~120 kDa), suggesting that dimerization domains are present in the C-terminal portion of cGI PDEs.

In this work we have characterized RcGIP1 and HcGIP2 recombinants and initiated structure/function studies relating to the N-terminal putative membrane-association domains. Future experiments will be directed to precisely define the domains involved in membrane association and intracellular localization and/or dimer or multimer formation as well as understanding structure/function relationships of other cGI PDE domains.

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