Identification of a major endogenous substrate for phospholipid/Ca^{2+}-dependent kinase in pancreatic acini as Gc (vitamin D-binding protein)

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A major 56 kDa substrate for phospholipid/Ca^{2+}-dependent kinase (C-kinase) in pancreatic acinar cells is physicochemically and immunologically indistinguishable from the vitamin D-binding protein, Gc or group-specific component. Cellular Gc was also phosphorylated in intact cells following treatment with carbachol as a physiological stimulus. These findings indicate the potential usefulness of Gc as a defined substrate for further studies of the biological role of C-kinase activity in pancreatic acini and possibly in other cells.

C-kinase *Pancreatic acini* *Gc protein*

1. INTRODUCTION

Phospholipid/Ca^{2+}-dependent protein kinase (C-kinase) has been implicated in the physiological responses of many cells to defined stimuli [1]. Further studies of the biological function of this enzyme have been hampered by difficulties in studying C-kinase activity in intact cells and the large number of unidentified protein substrates which have been reported [1]. Major 56 kDa C-kinase substrates have been found in several cell types, including peripheral blood lymphocytes [2,3] and pancreatic acinar cells [4,5]. In pancreatic cells, we now report the identification of the 56 kDa band as Gc or vitamin D-binding protein [6–9].

2. EXPERIMENTAL

Acinar cells were isolated, thoroughly washed and a soluble fraction obtained by sonication in 20 mM Tris-HCl, pH 7.5, containing 2 mM EDTA, 50 mM mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine and 100 μg/ml aprotinin [2,4,5]. Aqueous homogenates were obtained by centrifugation at 100000 × g at 4°C for 60 min and the supernatant collected. Endogenous protein phosphorylation was assessed as described [2,11,12] in 0.2 ml containing: 25 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 25 μM EDTA; sample protein (100–150 μg), 20 μM [γ-^{32}P]ATP (~4 × 10^7 cpm), in either the presence or absence of $CaCl_2$ and phosphatidylserine. Incubations were carried out for 3 min at 30°C and terminated by the addition of 0.1 ml stop solution containing 50 mM Tris-HCl, pH 7.5, 9% SDS, 15% glycerol and 0.05% bromophenol blue. Assays were also performed after treatment of intact cells with the physiological stimulus, carbachol. Acinar cells were pre-equilibrated with [^{32}P]orthophosphate in phosphate-free medium for 2 h at 37°C and stimulated with carbachol [4,5]. Cells were then washed, and phosphorylation performed as described above. Reactions were terminated by pelleting and addition of extraction/SDS buffer. For certain experiments, C-kinase was obtained in partially purified form [4,5] and Gc was purified chromatographically [13].

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SDS-PAGE and autoradiography [10,11] were carried out on phosphorylated samples using a 10% acrylamide separating gel [10]. Proteins were transferred to nitrocellulose by electroblotting [14], and examined sequentially with polyclonal rabbit antiserum to human Gc (which is known to cross-react immunologically with rat Gc) and peroxidase-conjugated anti-rabbit immunoglobulin [7]. For 2-dimensional electrophoresis, pancreatic extracts (100–150 μg) [4,5] were treated with sample buffer containing 1% Triton X-100 and 2% ampholytes. Isoelectric focusing was carried out on a pH gradient (3.0–7.0) followed by 10% SDS-PAGE [15]. Following transfer to nitrocellulose by electroblotting [13], samples were analyzed further both with specific antiserum to Gc and after onlay with ^{125}I-labeled G-actin [16].

3. RESULTS AND DISCUSSION

SDS-PAGE/autoradiography of a soluble extract from pancreatic acinar cells revealed the presence of a 56 kDa protein which demonstrated enhanced phosphorylation in the presence of added calcium (free concentrations 0.1–100 μM) and phosphatidylserine (fig.1a). Analysis of phosphorylated extracts from pancreatic acinar cells by SDS-PAGE, followed by electroblotting to nitrocellulose and incubation with polyclonal antisera specific to Gc, showed only one detectable band (fig.1b). This substrate shown in fig.1a also comigrated and reacted identically with Gc purified protein (fig.1b).

To provide further physicochemical identification of this major 56 kDa substrate, pancreatic extracts phosphorylated in the presence of Ca^{2+} and phosphatidylserine were subjected to 2-dimensional gel electrophoresis followed by electroblotting and incubation with Gc antibody. Upon color development, only one spot was visualized, coinciding with the 56 kDa substrate. This protein had a p*I* of approx. 4.8–5.1 (fig.2a), apparently identical to that of purified Gc [7,17]. Utilizing the same protocol, unphosphorylated extracts were also electroblotted and incubated with ^{125}I-labeled G-actin, which is known to bind with high affinity to Gc [6–9,16,17]. Autoradiography revealed a protein of identical molecular mass and p*I* which bound the radiolabeled probe (fig.2b). Since Gc is the only 56 kDa protein known to bind G-actin

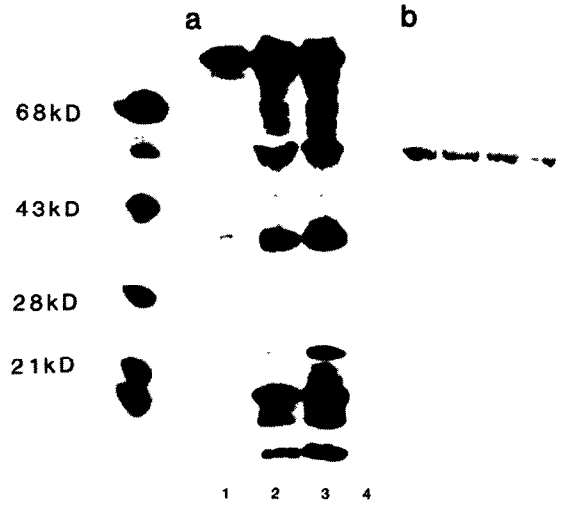

Fig.1. Detection of Gc protein in rat pancreatic acini. The marker proteins used for molecular mass determination were: bovine serum albumin, 68 kDa; Gc protein, 56 kDa; ovalbumin, 43 kDa; α-chymotrypsinogen, 28 kDa; soybean trypsin inhibitor, 21 kDa. (a) Autoradiogram of endogenous substrate proteins [1–3] and purified serum Gc [4]. Lanes: 1, no Ca^{2+} or phosphatidylserine; 2, 75 μM $CaCl_2$; 3, 75 μM $CaCl_2$ and 5 μg phosphatidylserine; 4, unphosphorylated purified Gc. (b) Immunoblot of the same gel.

under these conditions, these experiments provided a further criterion for unambiguous identification of this phosphorylated protein.

Further experiments were conducted to determine if purified Gc serves as a substrate protein for C-kinase by incubating Gc protein purified to homogeneity with purified C-kinase. SDS-PAGE/autoradiography revealed phospholabeling of Gc in a phospholipid/Ca^{2+}-dependent manner (fig.3a–c). In the absence of added Gc protein, no phosphoprotein band was detected at this position, effectively excluding the possibility that the phosphoprotein observed was an endogenous contaminant of the C-kinase preparation. Further studies were designed to determine if phosphorylation of the 56 kDa protein was associated with stimulation of acinar cells by a physiologic agonist. For these studies we undertook an in vivo/in vitro approach in demonstrating phosphorylation of the 56 kDa protein. Acini were treated with carbachol in vivo and subjected to in vitro phosphorylation conditions which would allow C-kinase to remain

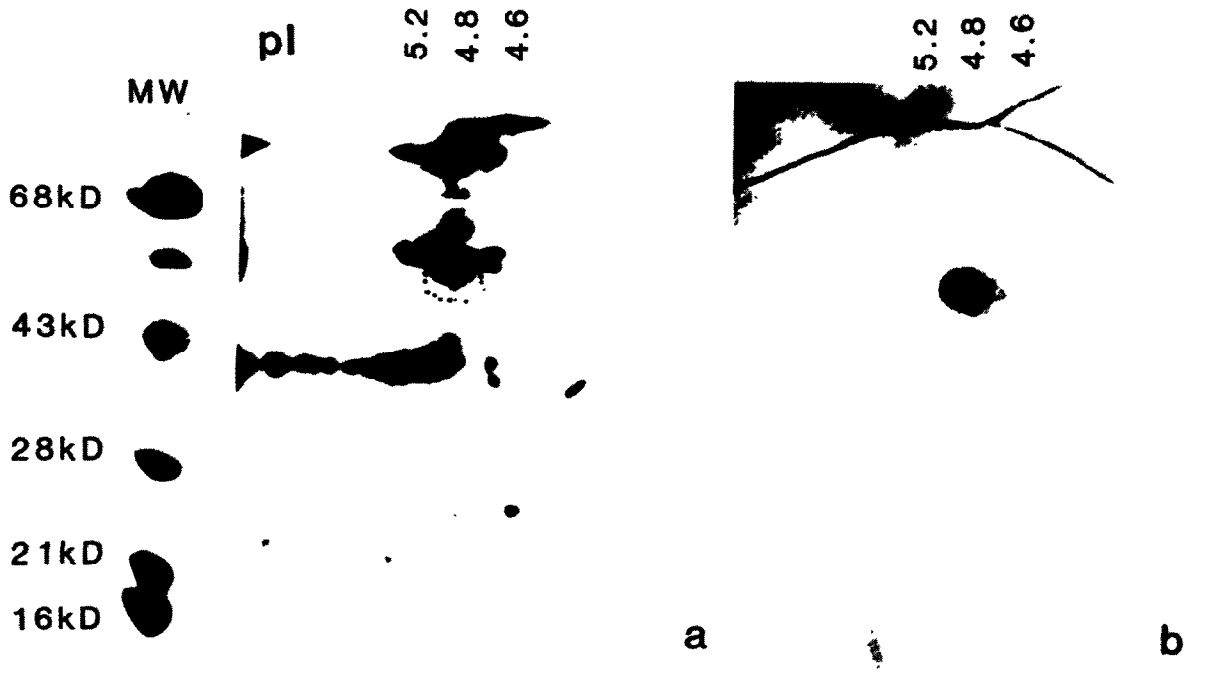

Fig.2. Autoradiograms of 2-dimensional SDS-PAGE of endogenously labeled pancreatic extracts and overlay with radioiodinated actin [11,15]. The p*I* marker proteins consisted of (p*I*): ovalbumin (4.6); bovine serum albumin (4.8); lactoglobulin (5.2). (a) Autoradiograms of 2-dimensional gels showing phosphorylation of Gc protein (circled for clarity) in the presence of $CaCl_2$ (75 μM) and phosphatidylserine (5 μg). (b) Electroblots of 2-dimensional gel of unphosphorylated samples were incubated in the presence of ^{125}I-labeled actin (3.5 μCi) and exposed for autoradiography. Note binding of radiolabeled actin to protein circled in (a).

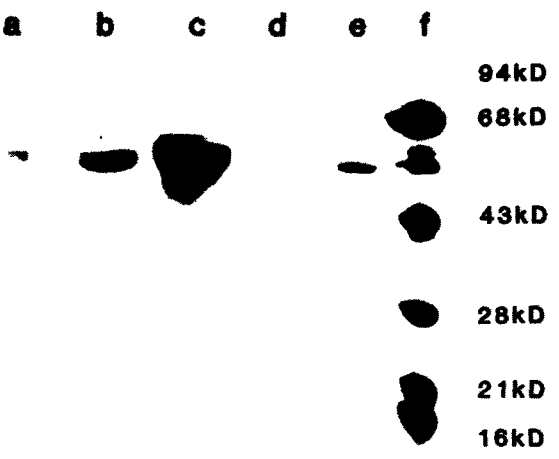

Fig.3. Autoradiogram (lanes a–d) of phosphorylated purified Gc protein from normal human serum. Homogeneous Gc1,2 phenotype [17] (20 μg) was incubated in the presence of purified C-kinase (10 μg) as in fig.1. Reactions were performed with and without $CaCl_2$ and phosphatidylserine (5 μg). Lanes: a, no added cofactors; b, $CaCl_2$ (75 μM); c, $CaCl_2$ (75 μM) plus phosphatidylserine (5 μg); d, no added C-kinase; e, Coomassie blue stain of purified Gc protein; f, molecular mass markers for fig.1, with purified Gc.

activated. This methodology, in our hands, has consistently reduced the signal-to-noise ratio of phosphorylations conducted in vivo. We were able to demonstrate that carbachol over a wide concentration range (fig.4, lanes a–g) stimulated the activation of C-kinase and thus dose-dependent increases in phosphorylation of 4 endogenous substrates (approx. 94, 56, 30 and 18 kDa). This consistently revealed increased phosphorylation of a 56 kDa protein, which was again indistinguishable from serum Gc, indicating that Gc might be a substrate for C-kinase in intact cells.

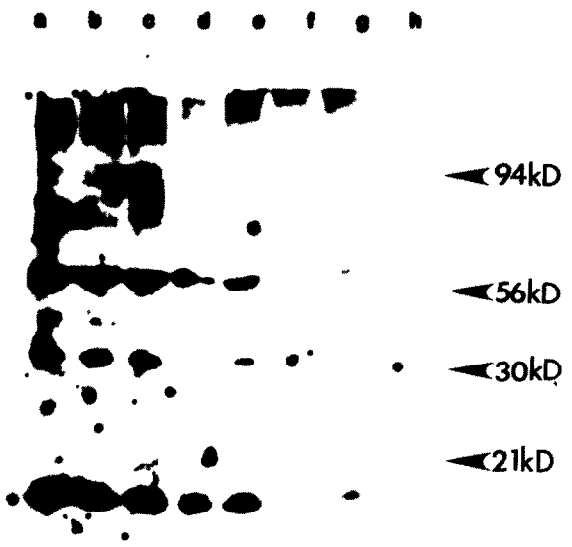

Fig.4. Pancreatic acini were treated at 37°C for 5 min in the presence or absence of carbachol. Cells were recovered by rapid centrifugation in an Eppendorf microfuge for 5 min at 15000 rpm. The cell pellet was immediately sonicated for 20 s in an ice-cold extraction buffer containing: 50 mM Tris, pH 7.5; 1 mM PMSF; 10 mM NaF; 10 mM $MgCl_2$; 2 mM benzamidine; 25 mM mercaptoethanol. Approx. 40 μl cellular extract, containing 150 μg protein, was recovered and to it 130 μl extraction buffer was added. Endogenous phosphorylation was carried out in a volume of 0.2 ml containing 20 μM [γ-^{32}P]ATP at 4°C for 10 min, boiled, followed by SDS-PAGE/autoradiography. Lanes: a–g, cells treated with carbachol (10^{-6}–10^{-12} M); h, control acini.

The above results demonstrate by both immunological and physicochemical criteria that the major 56 kDa protein substrate for C-kinase in pancreatic acini is indistinguishable from Gc protein under the experimental conditions employed. In addition, purified Gc and the 56 kDa pancreatic protein were both phosphorylated by purified C-kinase in a phospholipid/Ca^{2+}-dependent manner and increased phosphorylation of this protein occurred if intact cells were treated with a known secretagogue. While this 56 kDa protein did not serve as substrate for either cAMP- or cGMP-dependent protein kinase (not shown), we cannot exclude the possibility that it may be phosphorylated by other Ca^{2+}-dependent protein kinases. Nevertheless, the additional dependence of Gc phosphorylation upon phospholipid makes this unlikely, and we have shown dose-dependent inhibition of the 56 kDa protein phosphorylation in pancreatic acinar cells treated with polymyxin B, which exhibits substantially greater inhibitory action on C-kinase than calmodulin-dependent kinases [12].

This study raises questions regarding the potential role of Gc and its phosphorylation in modulation of pancreatic exocrine function: this protein is present in a wide variety of cell types [18,19], and in peripheral blood lymphocytes, evidence of involvement in signal transduction has been reported [6]. It will thus be of interest to explore interactions involving Gc and membrane components of pancreatic acini during receptor-mediated exocytosis and the possible modulation of this event through Ca^{2+} [20,21] and C-kinase [1,4,5]. Phosphorylation of Gc protein could also serve as a 'fingerprint' for the activation of C-kinase in intact cells, in a manner analogous to the 40 kDa substrate labeling observed upon activation of platelets by C-kinase mediated stimuli [1].

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