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Research Article

Analysis of a sub-proteome which co-purifies with and is phosphorylated by the Golgi casein kinase

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Abstract. In an attempt to gain information about the identity of the Golgi apparatus casein kinase(s) (G-CK), responsible for the phosphorylation of caseins in lactating mammary gland, the proteins present in fractions enriched in G-CK activity eluted from DEAE-Sepharose and heparin-Sepharose columns were resolved by two-dimensional electrophoresis and analyzed by mass spectrometry. This led to the identification of 47 proteins altogether, none of which is a bona fide protein kinase. At least 9 of the identified proteins however, are readily phosphorylated by co-purifying G-CK activity, and 7 are physically associated with it to give supramolecular complex(es) of about 500 kDa as judged from Superdex S200 gel fitration and glycerol gradient ultracentrifugation experiments. In contrast, the apparent molecular weight of G-CK estimated from an in gel activity assay after SDS-PAGE and renaturation is about 41 kDa. Many of the proteins phosphorylated by and/or associated with G-CK belong to the category of chaperonines, including HSP90, GRP-94 and -78, and various isoforms of protein disulfide isomerases, suggesting a global role of this kinase in the modulation of protein folding.

Key words. Casein kinase; Golgi apparatus; mammary gland; phosphoproteome; staurosporine; phosphoprotein; chaperone.

The first evidence of a phosphorylated protein was provided in 1883 by Olof Hammarsten who was able to show that bovine milk casein contains stoichiometric amounts of tightly bound phosphate [1]. Almost one century elapsed before the elucidation of primary structures of casein fractions began, revealing the presence of multiple phosphoserine (only sporadically phosphothreonine) residues, often clustered in triplets and invariably displaying an S-x-E/pS motif [2–6]. In the meantime, casein had been successfully employed as a model substrate for the detection and *in vitro* characterization of a variety of protein kinases whose characterization began in the 1960s and 1970s. Of historical interest was its use for the first detection of a protein kinase activity, in rat tissues [7], later shown to be accounted for by two classes of acidophilic and pleiotropic protein kinases now denoted by the acronyms CK1 and CK2 (casein kinase-1 and -2), reminiscent of their pronounced preference for casein over histones and other basic proteins as *in vitro* substrates [for a historical review see ref. 8]. It soon became clear, however, that none of these *in vitro* casein-phosphorylating kinases is responsible for the biosynthetic phosphorylation of casein fractions *in vivo*. This process takes place during casein secretion in the Golgi apparatus of the lactating mammary gland by the intervention of bona fide casein kinase(s) conventionally denoted by the acronym G-CK (Golgi casein kinase), which specifically recognizes the consensus sequence S-x-E/pS, which is unique among all known protein kinases and different

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from the consensus sequences of either CK1 (pS-x-x-S/T) or CK2 (S/T-x-x-E/D/pS) [9, 10]. This latter is differentiated by the position of the crucial acidic determinant which is at position n + 3 instead of being at n + 2 where it is strictly required for recognition by G-CK. Based on this information, a β-casein-derived peptide substrate specifically recognized by G-CK but not by CK2 or any other 'casein kinase' was developed [9] and has been successfully employed for monitoring G-CK in sources other than lactating mammary gland [11, 12]. An important outcome of these studies was the detection of G-CK in tissues where casein is not expressed, notably liver, brain, kidney, spleen and salivary gland [11, 13], leading to the concept that, similar to CK1 and CK2, G-CK is also not an enzyme dedicated only to the phosphorylation of casein, but is a pleiotropic kinase targeting a wide spectrum of endogenous substrates. This point of view was corroborated by a number of parallel observations implicating G-CK in the phosphorylation of several proteins other than casein, including, among others, osteopontin [14, 15], the salivary gland PRP-1 [13], GRP94 [16], aquaporin 2 [17] and p115 [18].

While the biochemical characterization of G-CK with special reference to the definition of its substrate specificity was quite straightforward thanks to the availability of fairly active G-CK preparations from the Golgi apparatus of lactating mammary gland and the synthesis of appropriate phosphoacceptor peptide substrates, the elucidation of the primary structure of G-CK turned out to be a troublesome and frustrating task. In fact, despite the recurrent efforts of several laboratories [11, 12, 19–26], G-CK could not be purified to homogeneity, a situation which has hindered up to now any reliable structural analysis of this elusive kinase. This prompted us to address the problem from a different angle, i.e. rather than further pursuing the purification of G-CK, to try instead to identify all the proteins which co-purify with G-CK activity by two-dimensional (2D) gel electrophoresis followed by mass spectrometry (MS) analysis. Although this approach has failed so far to identify the protein kinase responsible for G-CK activity, it has led to the identification of a supramolecolar complex that includes targets of G-CK and almost certainly the catalytic subunit of the kinase itself. These novel data, presented here, disclose new perspectives on the biological role of Golgi apparatus casein kinase and provide a reason for the difficulty in obtaining substantial amounts of it in pure form.

Materials and methods

Materials. [γ-^{32}P]-ATP was purchased from Amersham Biosciences. Peptides were synthesized as described elsewhere [9]. Other reagents were from Sigma.

Protein kinases. G-CK was purified from the Golgi fraction of lactating mammary gland according to Lasa et al. [11] and Brunati et al. [16] with some modifications. The Golgi suspension was obtained by ten cycles of freezing at –18 °C and thawing at room temperature. The Golgi crude extract was obtained by solubilization of Golgi suspension in extraction buffer supplied with 0.8 M NaCl and in extraction buffer supplied with 0.1% Triton X-100. CK1 and CK2 were purified from rat liver essentially as described elsewhere [27].

2D gel electrophoresis. Protein fractions were precipitated using methanol and chloroform [28] and solubilized in focusing buffer for 30 min (7 M urea, 2 M thiourea, 4% CHAPS, 3 mM TBP, 10 mM IAA, 0.2% carrier ampholytes pH 3–10). A solubilized sample (300 µl) was rehydrated and simultaneously loaded on the IPG strip (ReadyStrip IPG Strips, 17 cm, pH 3–10; BIO-RAD), at 50 V for 12 h. The voltage was increased to 10,000 V and focused for a total of 75,000 V/h using a Protean IEF Cell (Bio-Rad) [29]. Immediately after focusing, IPG strips were equilibrated in 6 M urea, 2% SDS, 0.375 M Tris, pH 8.8, 20% glycerol for 30 min. The second-dimension separation was run overnight on 10% SDS-PAGE using the Protean II XI Multi-Cell 2-D (Bio-Rad) at 20 mA per gel at 15 °C. After electrophoresis, gels were stained with colloidal Coomassie Brilliant Blue G-250 and destained with 5% v/v acetic acid.

In gel kinase activity assay. Aliquots of Golgi crude extract were electrophoresed according to Laemmli [30] in 10% acrylamide gels containing 0.5 mg/ml casein. After electrophoresis, SDS was removed by washing the gels twice with the washing buffer (50 mM Tris, pH 8.0, and 20% 2-propanol) for 30 min each at room temperature; the gels were then equilibrated for 1 h in buffer A (50 mM Tris and 5 mM 2-mercaptoethanol). Proteins were denaturated with 6 M guanidine HCl in buffer A (two 30-min changes), followed by an overnight renaturation at 4 °C in buffer A containing 0.04% Tween 20 (five changes). Subsequently, the gels were incubated for 60 min at room temperature in 10 ml of reaction buffer (50 mM imidazole, pH 7.0, 5 mM $MnCl_2$, 5 mM $MgCl_2$ and 10 mM ATP, 100 µM staurosporine ± 2 mM peptide substrate (28–40)) supplemented with 50 µCi of [γ-^{32}P]-ATP. The reaction was terminated by washing the gels with the bath solution (5% TCA and 1% sodium pyrophosphate). [γ-^{32}P]-ATP was removed by exhaustive washing of the gels in the bath. Finally, the gels were stained with CBB G-250, dried, and subjected to autoradiography.

Phosphorylation assays. Phosphorylation of proteins co-purifying with G-CK was performed by incubating the G-CK fraction from DEAE-Sepharose for 10 min at 30 °C in a mixture containing 50 mM imidazole, pH 7.0,

5 mM $MnCl_2$, 5 mM $MgCl_2$, 25 µM [γ-^{32}P]-ATP (specific activity 1500 cpm/pmol), 100 µM staurosporine. At the end of incubation, the samples were subjected to 2D PAGE and the radioactive spots were detected by autoradiography.

G-CK activity was assayed on α-casein or on the specific peptide substrate β(28–40) (0.5 mM) in the same conditions as described above except for the absence or presence of variable concentrations of staurosporine. CK1 and CK2 activities were assayed on α-casein or on the specific peptide substrates, RRRKDHDDEEDEAMSITA and RRRADDSDDDDD, respectively, as described previously [31]. α-Casein was resolved by SDS-PAGE, the corresponding radioactive bands were excised and the radioactivity determined in a liquid scintillation counter. ^{32}P incorporation in the peptides was evaluated by the phosphocellulose paper procedure [32].

Glycerol gradient centrifugation. The Golgi extract (350 µg) was loaded on top of an 11-ml linear gradient of glycerol (10–40%) made in 25 mM Hepes, pH 7.4, 1 mM EDTA, 0.5 M NaCl and 1 mM dithiothreitol. The tubes were centrifuged for 18 h at 30,000 rpm in a SW40 rotor (Beckman) at 4 °C. The tubes were fractionated into 12 fractions. Aliquots of each fraction were assayed for G-CK activity as described above. Thyroglobulin (669 kDa), apoferritin (443 kDa) and alcohol dehydrogenase (150 kDa) were used as standards for estimating the molecular weight (MW) of the complexes.

OptiPrep gradient centrifugation. Sub-cellular fractionation was performed using OptiPrep (Accurate Chemical and Scientific Company) gradient ultracentrifugation as described elsewhere [33]. A discontinuous gradient was prepared using 30, 25, 20, 15 and 10% OptiPrep dissolved in 50 mM Tris/HCl, pH 7.5, containing a protease inhibitor cocktail. Mammary gland (1 g) was homogenized in isotonic buffer and nuclei and cytosol were separated from the particulate fraction. The postnuclear particulate fraction was overlaid onto the discontinuous gradient and centrifuged at 100,000 g for 3 h at 4 °C. The gradient was removed in 15 equal fractions collected from the top of the gradient.

MS analysis. The spots obtained from 2D gels were manually excised and the protein digestion was performed in gel with porcine trypsin (Promega). The gel spots were shrunk with acetonitrile for 20 min and dried in a SpeedVac. About 5–10 µl trypsin (12.5 ng/µl in ammonium bicarbonate 50 mM) was added to each spot and the samples were kept at 4 °C for 45 min and then incubated overnight at 37 °C. The peptides were extracted by three changes of 50% acetonitrile/5% formic acid and the samples were then desalted using C18 ZipTip (Millipore). The digested proteins were analyzed by matrix-assisted laser desorption and ionization (MALDI)-MS using a M@ldi-HT (Waters). The protein digest was mixed with an equal volume of a solution of α-cyano-4-hydroxycinnamic acid (Sigma) (2.5 mg/ml in acetonitrile/0.1% formic acid 50/50) as matrix. A 1-µl sample of the mixture was spotted on a standard 96-well stainless steel MALDI target plate. The spectra were analyzed using Mascot engine search (Matrix Science) and PIUMS [34] (www.hh.se/staff/bioinf). The search was done against the rat database using a mass tolerance window of 50 ppm, carbamidomethylcysteine as fixed modification and oxidation of methionine as variable modification. The proteins were considered as correctly identified when both softwares gave the same identification with a p value <0.005 and at least 20% coverage of the sequence.

Results

G-CK accounts for the whole casein kinase activity of the Golgi apparatus and is abnormally insensitive to staurosporine. *In vitro*, casein is not phosphorylated by G-CK alone but also by several other protein kinases, in particular the two classes of ubiquitous and pleiotropic enzymes termed CK1 and CK2. We therefore wanted to assess if and to what extent protein kinases other than G-CK might account for the 'casein kinase' activity of the Golgi apparatus. For this, we took advantage of three peptide substrates developed for the specific monitoring of either CK1 or CK2 or G-CK [9], each being unaffected by the other 'casein kinases'.

As shown in table 1, neither a Golgi suspension nor a crude extract of it containing soluble G-CK displayed any significant activity toward the CK1- and CK2-specific peptides, while both readily phosphorylated, as expected, the G-CK-specific peptide. It may be noted, incidentally, that G-CK is quantitatively extracted by our procedure,

Table 1. Determination of Golgi casein kinase activity revealed using specific peptide substrates for G-CK (KKKIEFQSEEQQQ), CK1 (RRKDLHDDEEDEAMSITA) and CK2 (RRRADDSDDDDD).

	Specific peptide phosphorylation (pmol min^{-1} mg^{-1})		
	G-CK	CK1	CK2
Golgi suspension	3221 ± 132 (SD)	9.58 ± 1.08 (SD)	12.36 ± 1.12 (SD)
Golgi crude extract	3262 ± 150 (SD)	6.45 ± 0.87 (SD)	9.98 ± 0.98 (SD)

Golgi suspension and Golgi extract were obtained and assayed as described in Materials and methods.

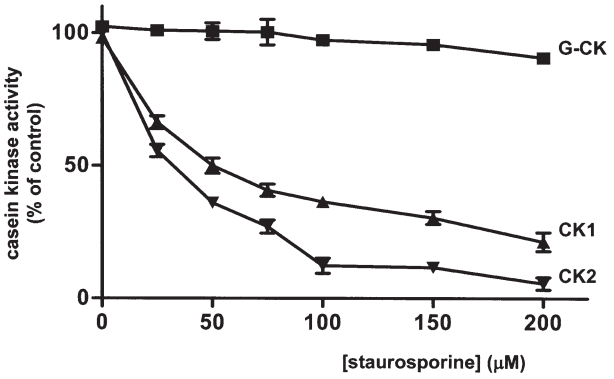

Figure 1. Effect of increasing concentration of staurosporine on casein phosphorylation by G-CK (■), CK1 (▲) and CK2 (▼). Protein kinases were assayed using α-casein as phosphorylatable substrate. The incubation time was 10 min as described under Materials and methods. Casein kinase activity is expressed as the percentage of the control values obtained in the absence of staurosporine. Values represent means of four separate experiments, with the SE indicated by vertical bars.

ruling out the possibility that only a minor fraction of G-CK is solubilized from the membranes.

In the course of these experiments, we tested a large variety of protein kinase inhibitors in the G-CK assay, with the aim of developing G-CK inhibitors useful for functional studies. All the inhibitors tested, however, including a large panel of flavonoids (quercetin, apigenin, luteolin, fisetin, miricetin), an ample spectrum of CK2 inhibitors and related compounds derived from the scaffolds of emodin [35] and tetrabromobenzimidazole [36], several pyrimidine derivatives including olomoucine and roscovitine, which are potent inhibitors of cyclin-dependent kinases, several inhibitors of tyrosine kinases (PP2, tyrfostine, genistein, piceatannol, AG 490, AG 1200, AG 1478), proved entirely ineffective on G-CK (data not shown). Especially striking is insensitivity to staurosporine, a broad-specificity inhibitor suppressing the activity of most protein kinases in the low-nanomolar range. Even so-called 'staurosporine-resistant' protein kinases (including among others CK1 and CK2) [37] are inhibited if staurosporine is raised to micromolar concentrations (with an IC_{50} between 1 and 30 μM). By sharp contrast and unlike CK1 and CK2, G-CK is fully unaffected by staurosporine up to 200 μM (fig. 1). We took advantage of this unique property of G-CK to see whether and to what extent the casein-phosphorylating activity of Golgi crude extracts was inhibited by 100 μM staurosporine. We found no detectable inhibition (data not shown), consistent with the concept that casein phosphorylation by Golgi extracts is entirely accounted for by G-CK activity.

2D analysis of proteins that co-purify with G-CK activity. The Golgi apparatus of rat lactating mammary glands was isolated by sucrose gradient ultracentrifugation and G-CK activity was partially solubilized as described in Materials and methods (table 1). The extract was subjected to DEAE-Sepharose column chromatography and G-CK was monitored using the specific peptide substrate β-casein (28–40) (fig. 2A). The G-CK peak, eluted by 0.3 M NaCl, was collected as indicated in figure 2A and subjected to heparin-Sepharose column chromatography where the main peak of G-CK eluted with 0.3 M NaCl (fig. 2B). A minor peak of activity eluting with about 0.55 M NaCl was also generally observed, possibly reflecting heterogeneity of the kinase, as also suggested by others [12]. The more retarded peak, however, underwent rapid inactivation and was not further investigated.

The G-CK fraction from DEAE-Sepharose was subjected to 2D gel electrophoresis and the gel was stained with colloidal Coomassie Brilliant Blue (fig. 3). Spots were

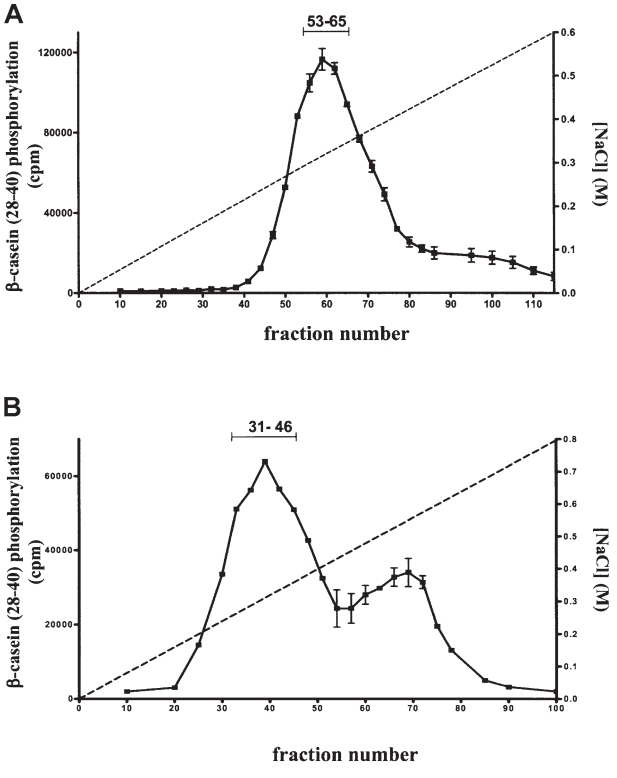

Figure 2. Partial purification of mammary gland G-CK by DEAE-Sepharose (*A*) and heparin-Sepharose (*B*). (*A*) Golgi crude extract was loaded onto a DEAE-Sepharose column and bound proteins were eluted using an NaCl gradient (broken line). Fractions were collected and assayed for G-CK activity. The active fractions were pooled as indicated. (*B*) Pooled fractions from the DEAE-Sepharose column were applied to a heparin-Sepharose column, and bound proteins were eluted with an NaCl gradient (broken line). Fractions were collected and assayed for G-CK activity. The active fractions were pooled as indicated. Reported values represent means of four separate experiments, with the SE indicated by vertical bars.

Table 2. Proteins co-purifying with G-CK activity on three sequential steps.

Spot No.	Swiss-Prot accession number	Protein name	Purification step			MW (kDa)	pI
			DEAE-Sepharose	heparin-Sepharose	Superdex S200		
1	P16086	spectrin alpha chain	+			284.00	5.20
2	P12785	fatty acid synthase	+			273.00	5.96
3	Q9JLA3	UDP-glucose: glycoprotein glucosyltransferase 1	+			174.00	5.39
4	Q63617	150-kDa oxygen-regulated protein	+			111.30	5.11
5, 6	Q62839	GM130	+			111.40	4.88
7	AAH61788	alpha actinin 4	+			104.90	5.27
8	O88600	ischemia responsive 94-kDa protein	+			94.00	5.13
9	Q8BHN3	glycoside hydrolase	+			106.80	5.67
10, 22	P52873	pyruvate carboxylase	+			129.60	6.25
11	P08113	GRP94	+	+	+	92.40	4.74
12	AAH61788	alpha actinin 4	+			104.90	5.27
13	P46462	transitional endoplasmic precursor	+	+		89.30	5.14
14	P34058	HSP-90	+	+		83.00	5.06
15	Q921X2	similar to PKC substrate	+			58.80	4.41
16	Q9R0T4	epithelial cadherin precursor	+			98.70	4.67
17, 18	P06761	BiP	+	+	+	72.30	5.07
17	P38659	PDI A4	+	+	+	72.80	4.99
18	Q7TNY6	DMT1-associated protein	+	+		60.30	4.99
19, 20, 21	P48037	annexin VI	+	+	+	75.60	5.39
19	P08109	heat shock cognate 71-kDa protein	+		+	70.80	5.37
23, 24	P18163	long-chain fatty acid CoA-ligase	+			78.00	6.60
25	P19226	60-kDa heat shock protein	+			60.90	5.91
26	Q63083	nucleobindin 1 precursor	+			53.50	5.04
27	Q5XIF6	similar to tubulin alpha-4 chain	+			49.90	4.95
28	P18418	calreticulin	+	+		48.00	4.33
29	P04785	PDI	+	+		57.00	4.82
30, 31	Q9JI85	nucleobindin 2 precursor	+	+		50.00	5.02
31, 32, 33	P10719	ATP synthase beta chain	+	+		56.40	5.18
31, 33	Q63081	PDI A6	+	+	+	48.00	5.00
34	Q9D1Q6	Erp 44	+			47.00	5.09
35	P07335	creatine kinase beta chain	+			42.70	5.33
36	P02571	actin	+	+		41.80	5.31
37	P38983	40S ribosomal protein	+	+		32.70	4.80
38	O35783	calumenin precursor	+	+		37.00	4.40
39, 40	P12007	isovaleryl CoA dehydrogenase	+			46.40	8.30
41, 42	P02661	alpha casein precursor	+	+	+	31.80	5.32
43	P15999	ATP synthase alpha chain	+			58.80	9.22
44	P49432	pyruvate dehydrogenase E1 component	+			38.80	5.94
45	Q63610	tropomyosin isoform 6	+			29.00	4.75
46	P42655	14-3-3 protein epsilon	+			29.20	4.63
47	P35215	protein zeta/delta	+			27.80	4.73

numbered as indicated, excised and the proteins identified by peptide mass fingerprinting experiments as described in Materials and methods. This led to the identification of 47 individual proteins, listed in the third column of table 2 with the conventional identification number adopted in figure 3, the corresponding SwissProt code number, their isoelectric points and molecular weights. Note that in some cases, the same protein (e.g. BiP/GRP78, annexin VI, PDIA6) was identified in distinct spots, probably due to limited proteolysis and/or other post-translational modifications, while, conversely, a few spots (notably No. 17, 18, 19, 31, 33) gave rise to two distinct proteins. None of the identified proteins is a bona fide protein kinase.

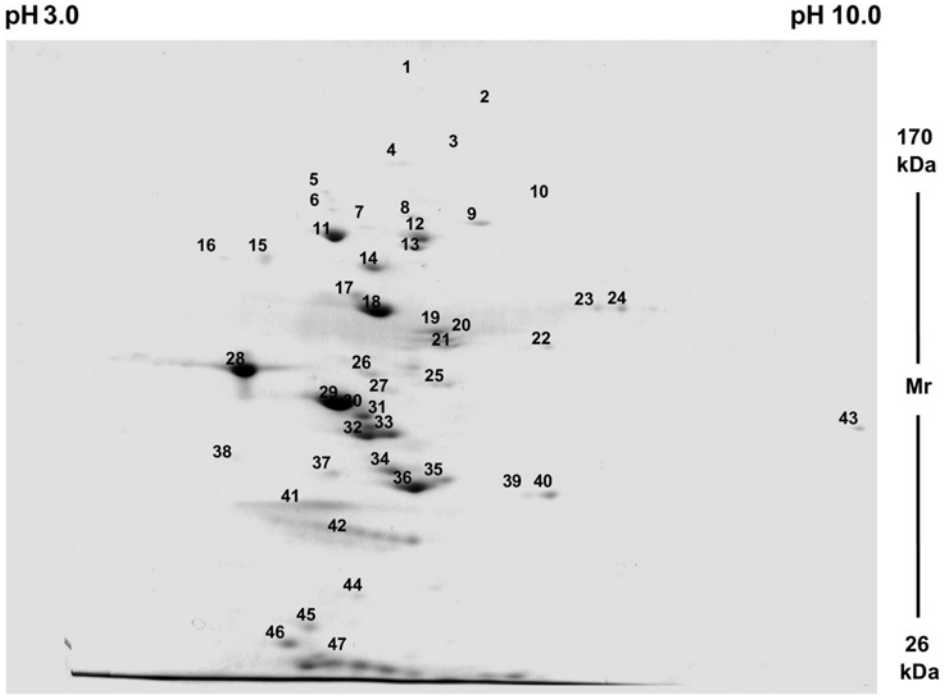

Figure 3. 2D gel analysis of proteins that co-elute with G-CK activity, on DEAE-Sepharose. Proteins (300 µg) were resolved by IEF on an immobilized pH 3–10 gradient in the first dimension. They were then separated by SDS-PAGE in the second dimension, as described in Materials and methods. Finally, the proteins were detected with colloidal Coomassie Brilliant Blue staining. All detectable spots were labeled from 1 to 47 and subjected to MS analysis.

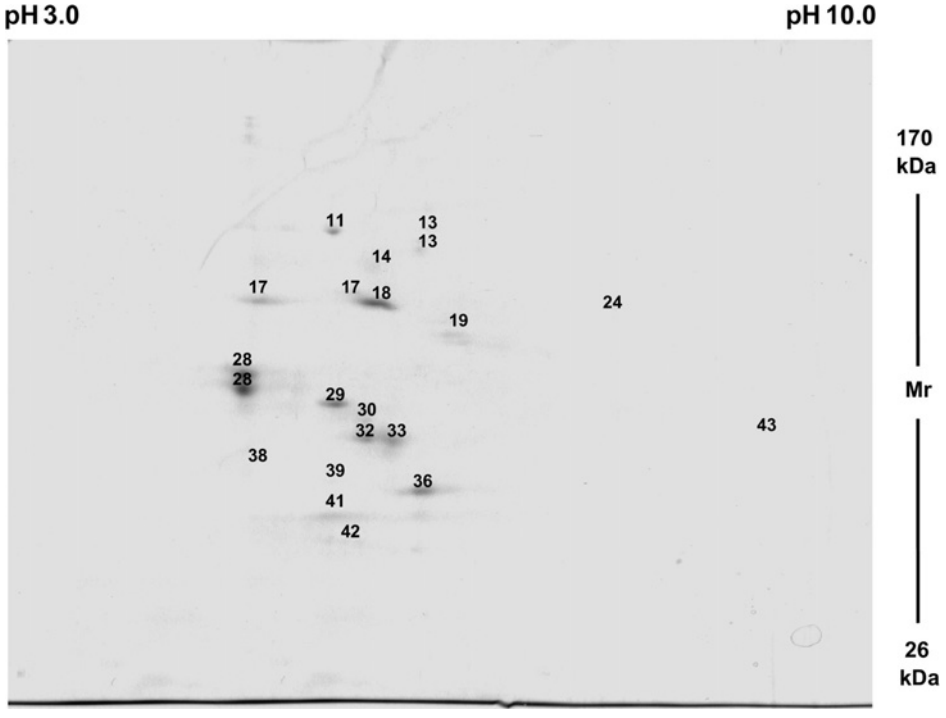

Figure 4. 2D gel analysis of proteins that co-elute with G-CK activity on heparin-Sepharose. Proteins (300 µg) were resolved by IEF on an immobilized pH 3–10 gradient in the first dimension and by SDS-PAGE in the second dimension, as described in Materials and methods. The gel was stained with colloidal Coomassie Brilliant Blue. Spots were labeled using the same numeration as in figure 3 and subjected to MS analysis.

By performing the same 2D analysis on the main G-CK fraction eluted from heparin-Sepharose, the pattern displayed in figure 4 was obtained. The subsequent MS analysis revealed that in this case also, some proteins were present in different spots while some spots contained two different proteins. This led to the identification of 18 individual proteins altogether (table 2, column 5) all of which had already been found in the DEAE-Sepharose fraction.

G-CK participates in multimolecular complexes. Co-elution of many proteins, displaying in some cases different isoelectric points, with G-CK activity upon two subsequent chromatographic steps raised the possibility that at least some of these might form stable complexes with G-CK. To gain more information on this point, two experimental approaches were exploited. First, the heparin-Sepharose fraction was subjected to gel filtration through a Superdex S200 column. As shown in figure 5A, all the G-CK activity was eluted as a sharp and symmetric peak overlapping an absorbance peak whose apparent MW was around 500 kDa. This peak still contained a number of proteins, as revealed by 2D gel electrophoresis (fig. 5B). All the Coomassie spots, with just three exceptions, could be identified by MS analysis and were found to coincide with proteins which were also found in the heparin-Sepharose and/or in the DEAE-Sepharose fractions (table 2, column 6). In this case, also, no bona fide protein kinase could be detected, despite the presence of remarkable G-CK activity in the fraction. Note that none of the proteins identified in the 500-kDa fraction eluted from Superdex S200 displayed per se a MW compatible with such a large size, the largest one, GRP94, having a molecular mass of 92 kDa. This corroborates the view that co-elution reflects the formation of discrete supramolecular complexes.

Similar conclusions were reached employing a different approach based on glycerol gradient ultracentrifugation of the crude Golgi extract (fig. 6A). In this case G-CK activity migrated as a rather broad, yet substantially symmetrical fraction peaking around 500–600 kDa, as judged from calibration with proteins of known MW. This fraction, collected as indicated in figure 6A and chromatographed through a DEAE-Sepharose column to remove the bulk of contaminating proteins, was submitted to 2D gel electrophoresis (fig. 6B) revealing a number of spots (denoted by arrows) also found in the 2D pattern of the fraction eluted from Superdex S200, notably those corresponding to GRP94, BiP/GRP78, PDIA4, HSP71 and annexin 6 and PDIA6. Again, since the MW of these proteins varies between 92 and 48 kDa, their recovery in a fraction exhibiting >500 kDa MW upon ultracentrifugation is symptomatic of supramolecular organization.

Since this fraction is primarily characterized by its G-CK activity, we wanted to gain information about the molecular mass of the kinase(s) responsible for such an activity. Given the failure to identify the kinase at the protein level even in partially purified fractions (e.g. Superdex S200; see above), we decided to perform an in gel casein kinase assay after SDS-PAGE and renaturation, taking advantage of the knowledge that casein kinase activity in our Golgi preparation is only due to G-CK (see above). This was performed using as G-CK source the same crude Golgi extract also used for the ultracentrifugation experiment of figure 6A. As shown in figure 7, a single clear band of casein kinase activity could be detected in the gel, with an apparent MW around 41 kDa. The intensity of ca-

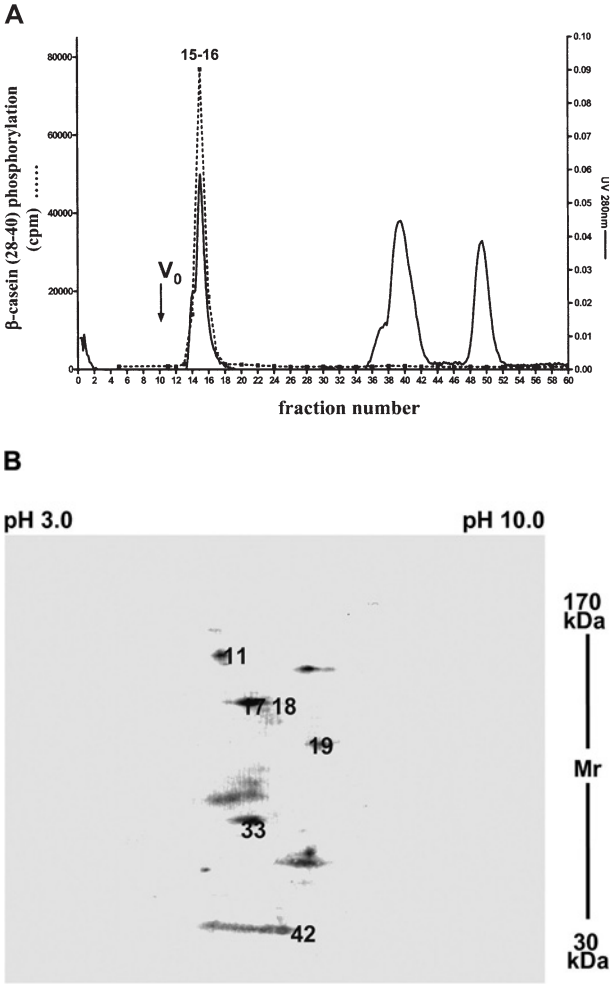

Figure 5. Identification of proteins that associate with G-CK activity upon Superdex S200 gel chromatography. (*A*) Absorbance and activity profiles: pooled fractions from heparin-Sepharose were loaded onto a Superdex S200 column and proteins were eluted with equilibration buffer plus 0.5 M NaCl. Active fractions were pooled as indicated. (*B*) 2D gel analysis of the 15–16 fractions from Superdex S200: proteins were first separated by IEF on an immobilized pH 3–10 gradient according to their pI, and then by SDS-PAGE according to their molecular masses, as described in Materials and methods. The gel was stained with colloidal CBB and the visible spots were subjcted to MS analysis.

sein phosphoradiolabeling was not decreased by 100 µM staurosporine, while it was suppressed by adding in the reaction a large excess (2 mM) of the specific G-CK peptide substrate. This leaves no doubt that the 41-kDa kinase responsible for in gel casein phosphorylation is indeed G-CK. Note that in a parallel experiment run with a gel without casein, Coomassie staining did not reveal any detectable protein band in the position to which G-CK activity migrated (fig. 7, lane 4). This may well account for the failure to identify any protein kinase among the spots analyzed by MS, due to the fact that its minimal amount escaped detection. This would also argue in favor of an unusually high specific activity of G-CK and reinforce the scepticism about the real degree of purity of currently available G-CK preparations [12].

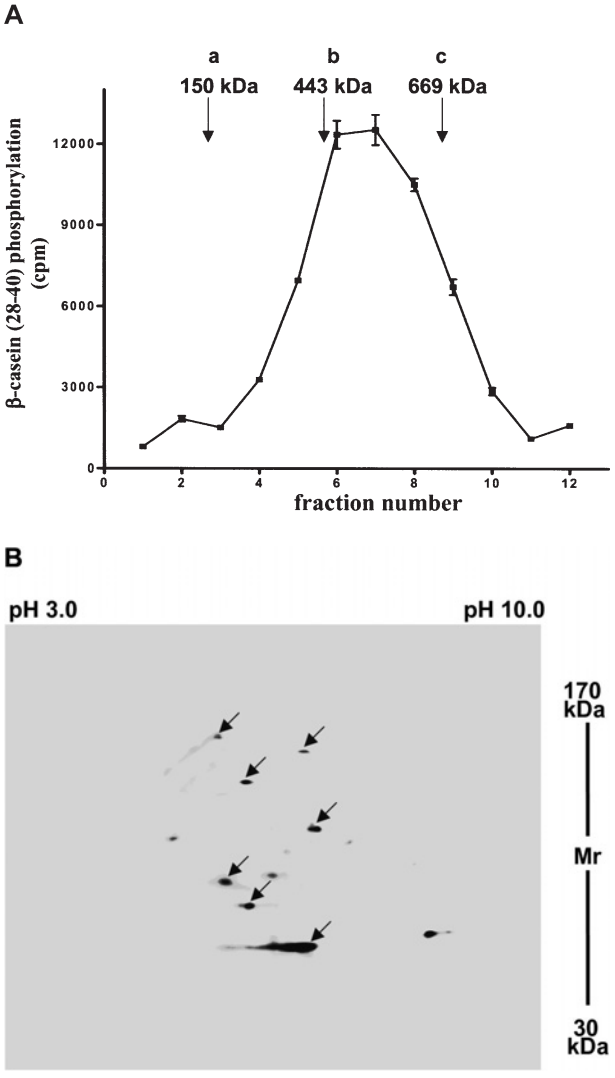

Figure 6. G-CK activity and protein profiles after glycerol gradient ultracentrifugation. (*A*) Golgi extract was loaded on top of a linear gradient of glycerol (10–40%) and centrifuged for 18 h at 30,000 rpm in a Beckman SW40 rotor. The fractions (1 ml) were collected from the top and assayed for G-CK activity. Active fractions were pooled as indicated. Reported values represent means of four separate experiments, with the SE indicated by vertical bars. The position of proteins used for standard molecular masses is also indicated: alcohol dehydrogenase (a) (150 kDa), apoferritin (b) (443 kDa), thyroglobulin (c) (669 kDa). (*B*) Pooled fractions from the glycerol gradient were applied to a DEAE-Sepharose column, and bound proteins were eluted by NaCl gradient as in figure 2A. Fractions were collected and assayed for G-CK activity. Active fractions were pooled and subjected to 2D PAGE as described in Materials and methods: proteins were first separated by IEF on an immobilized pH 3–10 gradient according to their pI, and then separated by SDS-PAGE in the second dimension. The gel was stained with colloidal CBB.

Identification of endogenous protein substrates of G-CK. While the experiments described above support the view that a number of proteins co-purifying with G-CK are physically associated with it, they did not provide any information about the functional relatedness between these proteins and G-CK. To shed light on this point we wanted to see if some of these proteins could be phosphorylated by G-CK. The G-CK fraction eluted from DEAE-Sepharose (collected as indicated in figure 2A) was incubated with [γ-^{32}P]-ATP and Mg^{2+}/Mn^{2+} ions and the radiolabeled proteins were resolved by 2D gel electrophoresis, revealed by autoradiography (fig. 8) and analyzed by MS to try to identify all of them. Note that their

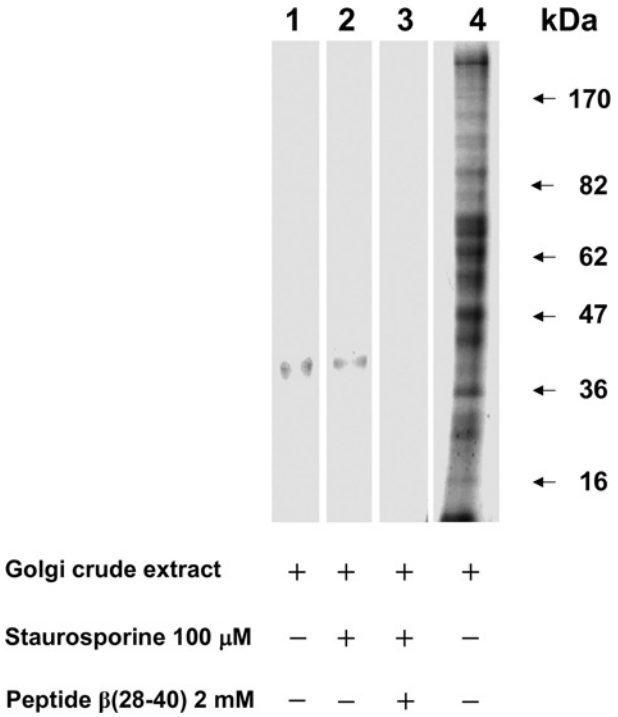

Figure 7. In gel casein kinase assay of Golgi crude extract in different conditions. Golgi extract (20 µg) was subjected to an in gel kinase assay with casein as substrate, as detailed in Materials and methods, either without inhibitors (lane 1), or in the presence of 100 µM staurosporine (lane 2), or 100 µM staurosporine and 2 mM specific G-CK peptide substrate β (28–40) (lane 3). Lane 4 shows the Coomassie staining of the same sample subjected to SDS-PAGE under identical conditions but in the absence of casein.

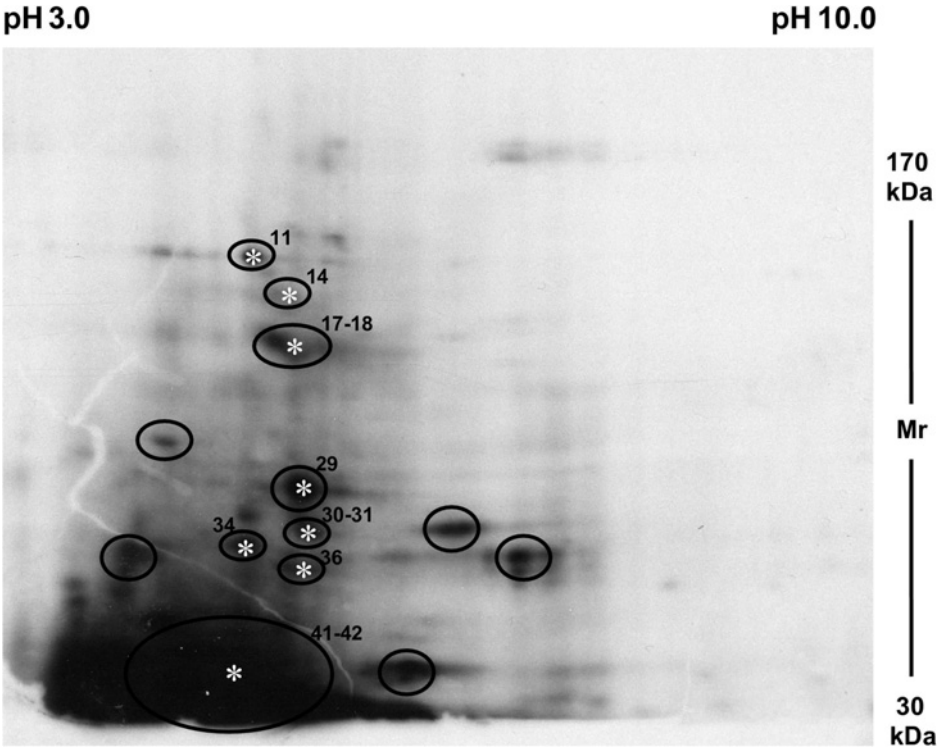

Figure 8. 2D resolution of proteins which are phosphorylated by G-CK in the fraction eluted by DEAE-Sepharose. The DEAE-Sepharose fraction displaying G-CK activity (see fig. 2A) was incubated with γ-^{32}P-ATP and resolved by 2D electrophoresis as described in Materials and methods. The gel was then subjected to autoradiography. All the circled spots were excised and subjected to MS analysis. Circled spots with an asterisk were identified and labeled according to the numbering adopted in figure 3A. Circled spots without an asterisk led to no or low-quality mass spectra that did not allow protein identifications.

phosphorylation pattern was not altered by 100 µM staurosporine, while being adversely affected by the addition of the G-CK-specific peptide substrate (not shown), confirming the involvement of G-CK in the phosphorylation of all of these proteins.

However, all the radioactive spots circled in figure 8 were excised and analyzed by MS; only those denoted by an asterisk could be identified, due to an insufficient amount of protein in the other radiolabeled spots, many of which were actually undetectable by Coomassie and silver staining.

As expected, the huge radiolabeled spot occupying the bottom left corner of the electropherogram corresponds to variably phosphorylated forms of casein. The other nine identified phosphoradiolabeled proteins are listed in table 3, which also shows that all of them contain one or more canonical consensus sequences (S-x-E) specifically recognized by G-CK.

As expected, all the identified phosphorylated proteins were also present in the Golgi proteome co-eluting with G-CK from DEAE-Sepharose (table 2, column 4), this also representing the source for the phosphorylation experiment. More interestingly, all of them, with just two exceptions (nucleobindin 2 precursor and Erp44) were

Table 3: Identification of proteins phosphorylated by G-CK, and consensus sequences.

Spot No.	Identified protein	Consensus sequence
11	GRP94	GKSREGS SDSNEFS RPSKEVE SFSKESD NVSRELQ KESREAT KESTEKD
14	HSP90 beta	VGSDEED GESKEQV
17, 18	BiP	KLSPEDK
17	PDI A4	RLSQELD TLSEEKR PDSWETL
18	DMT1-associated protein	PDSEERP DTSPEVG NMSKEDA
29	PDI	VDSSEVT PESDELT
30, 31	nucleobindin 2 precursor	RLSQELD TLSEEKR PDSWETL
34	ERp44	TESLEIF
36	actin	SSSLEKS

also among the proteins co-eluting with G-CK from heparin-Sepharose, and three (GRP94, BiP/GRP78, PDIA4) participated in the supramolecular complex eluted from Superdex S200 with G-CK activity. Moreover, some of the other proteins found in the Superdex S200 fraction may have been among those radioactive spots which could not be identified (circles without asterisk, in figure 8).

To validate the concept that association of G-CK with its substrates is not an artifact arising from the purification procedure but indeed reflects co-localization of the proteins in the Golgi apparatus, sub-cellular particles were resolved by OptiPrep gradient ultracentrifugation and analyzed for the presence of G-CK activity and GRP94 and GRP78 immunodetected by Western blot. As shown in figure 9 all the G-CK activity was recovered in the Golgi, whereas, as expected, GRP94 and GRP78 were predominant in the microsomal fraction. A substantial amount of both GRP94 and GRP78 however did co-localize with G-CK activity in the Golgi fraction.

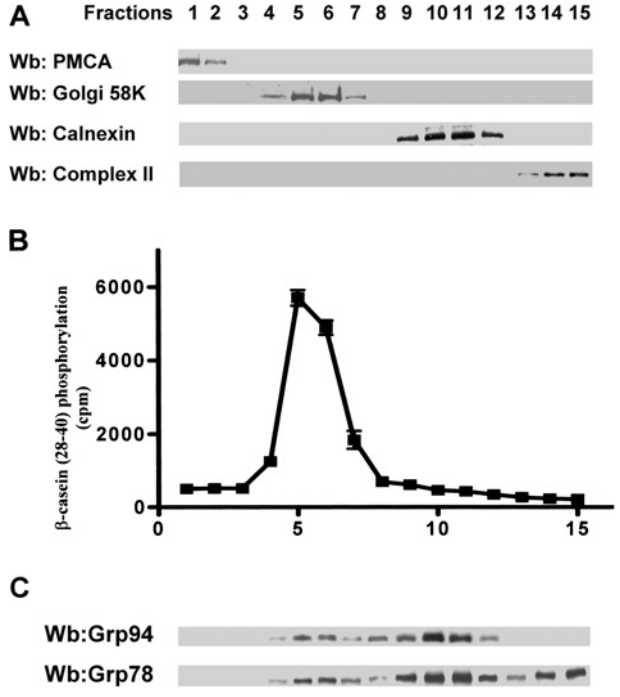

Figure 9. Subcellular localization of G-CK, GRP94 and GRP78 in lactating mammary gland. Mammary gland (1 g) was homogenized in isotonic buffer and nuclei and cytosol were separated from the particulate fraction. The latter was then fractionated by ultracentrifugation on a discontinuous OptiPrep gradient to separate the cellular organelles, as described in Materials and methods. (*A*) Aliquots of the resulting fractions were immunoblotted with the following organelle-specific antibodies: anti-PMCA (plasma membrane), anti-Golgi 58K protein (Golgi apparatus), anti-calnexin (endoplasmic reticulum) and anti-complex II (mitochondria). (*B*) Aliquots of the fractions were assayed for G-CK activity. (*C*) Aliquots of the fractions were immunoblotted with either anti-GRP94 or anti-GRP78 antibodies, as indicated.

Discussion

The data presented provide a functional characterization of the Golgi apparatus casein kinases of lactating mammary gland based on the identification of a panel of proteins which beside co-purifying with G-CK activity are physically associated with and/or phosphorylated by the kinase itself.

The failure to identify among these proteins any kinase which could account for G-CK activity, albeit disappointing, did not entirely come as a surprise, given the absence of any bona fide protein-phosphorylating enzyme in the most exhaustive Golgi proteome available, listing more than 400 proteins [38].

In light of the above considerations, two possibilities are open: (i) G-CK activity is a catalytic process performed by a protein of unknown function or an ancillary property of proteins classified in functional categories unrelated to eukaryotic protein kinases and possibly among those identified in this work; (ii) G-CK is indeed a bona fide protein kinase but its levels are too low for detection even in the samples most enriched in its activity.

While the unique insensitivity to staurosporine, unmatched among canonical protein kinases and only shared to the best of our knowledge by Bud32, an atypical protein kinase [39], may argue in favor of profound structural diversities in the catalytic site of G-CK compared to canonical protein kinases, in gel detection of its activity in a region where no Coomassie band(s) are visible (see fig. 7) supports the view that G-CK is indeed a minor component of the Golgi proteome even in a context, like that of lactating mammary gland, where its expression should be maximal.

Novel information provided by our present work is that a subunit displaying in gel G-CK activity migrates in SDS-PAGE with an apparent MW around 41 kDa. This would still be consistent with an average catalytic domain of a canonical eukaryotic protein kinase, whose minimal size approximates 30 kDa but is generally increased by the presence of insertions and/or regulatory domains. While, according to our in gel experiments, the 41-kDa subunit is still able to catalyze casein phosphorylation, such a low-MW free subunit is not detectable under non-denaturing conditions as we constantly find all G-CK activity associated with a multimolecular complex of about 500 kDa, as judged from either Superdex S200 gel filtration after two purification steps (DEAE and heparin-Sepharose) (fig. 5A) or glycerol gradient ultracentrifugation of the crude Golgi extract (fig. 6). A number of proteins associated with such multimeric complex(es) displaying G-CK activity, namely GRP94, BiP/GRP78, PDIA4, HSP71, annexin 6 and PDIA6, have been identified: three of these (GRP94, BiP/GRP78, PDIA4) are also phosphorylated by G-CK. Whether any of these and/or the other associated proteins which are not phosphory-

lated operate as regulators of G-CK activity remains an open question.

Beside the aforementioned protein substrates which are present in the 500-kDa complex co-eluting with G-CK activity from Superdex S200, many other proteins among those co-eluting with G-CK from DEAE-Sepharose were phosphorylated by G-CK, corroborating the concept that this kinase is not an enzyme dedicated to the phosphorylation of casein, but a pleiotropic protein kinase. Several of these polypeptides readily phosphorylated by endogenous G-CK to give rise to intense radioactive spots upon 2D gel electrophoresis could not be identified due to the paucity of protein present in the radioactive spots. Identification was possible of GRP94, BiP/GRP78, PDIA4, HSP90, DMT1-associated protein, PDI, nucleobindin 2 precursor, Erp44 and actin. Interestingly, all these protein substrates with the exception of two (Erp44 and nucleobindin 2 precursor) were also detected among the proteins co-eluting with G-CK from heparin-Sepharose, and at least three of them (GRP94, BiP and PDIA4) are present in the 500-kDa G-CK complex eluted from Superdex S200.

Noteworthy is that almost all the proteins associated with and/or phosphorylated by G-CK are chaperones implicated in the attainment and/or maintenance of proper protein folding: two are heat shock proteins (HSP90 and HSP71), two are glucose-related proteins (BiP/GRP78 and GRP94) and three (PDI, PDIA4, PDIA6) are protein disulfide isomerases required for the disulfide-coupled folding of proteins. Erp44 also appears to be involved in the control of oxidative protein folding [40], while the inclusion of DMTI-associated protein among the G-CK targets is of special interest since this protein, also termed GCP60-PAP7, plays a crucial role in the maintenance of the Golgi structure [41] and has already been described as a prominent Golgi phosphoprotein, whence its synonym Golgi phosphoprotein 1. Some of these proteins are generally held to be components of the endoplasmic reticulum rather than of the Golgi apparatus; we have shown, however, that a substantial sub-fraction of GRP94 and GRP78 localizes to the Golgi apparatus (see fig. 9). Note, moreover, that GRP94 and Erp44 were also found in the Golgi proteome described by Wu et al. [38], and nucleobindin has been reported to interact with Gia3 in Golgi [42]. This latter observation discloses a link with another G-CK substrate, DMT1-associated protein/GCP60, which also interacts with members of the giantin family. Even more interesting is the interaction occurring between GRP94 and PDIA4 [43], since the intimate relatedness of these two proteins with G-CK is supported by two observations: both are phosphorylated by G-CK and both participate in the supramolecular complex eluting together with G-CK from Superdex S200.

The physical association of G-CK with chaperones may represent a device to keep the kinase in its fully active conformation preventing premature unfolding and degradation. The intracellular activity of several protein kinases is critically regulated by mechanisms of this kind, a notable example being that of the control of a battery of growth- and apoptosis-related kinases such as Aurora, Akt and v-Src by HSP90 in association with the co-chaperone Cdc37 [44]. This may also lead to a loss of activity of G-CK in parallel with the removal of chaperones, ultimately accounting for the difficulty to obtain active preparations of the purified kinase.

In a different vein, the observation that many chaperones and refolding proteins are phosphorylated by G-CK discloses the complementary possibility that this pleiotropic kinase plays a general role in their regulation, being ultimately committed to a ‘supervision’ of protein folding and renaturation in the Golgi apparatus under basal conditions. It should be born in mind in this connection that the Golgi apparatus is committed to a variety of cellular functions among which of crucial relevance is the processing of proteins which are going to be secreted into the extracellular fluid. It is essential that, before leaving the cell, these proteins are correctly refolded in their biologically active conformation, a task to which a variety of chaperones are committed, whose functional efficiency might be subjected to supervision by G-CK. Further experimentation and in particular the still missing molecular characterization of the Golgi casein kinase(s) are required to validate this working hypothesis.

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