GRP94 (endoplasmin) co-purifies with and is phosphorylated by Golgi apparatus casein kinase

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Abstract A phosphorylatable protein band of about 94 kDa (as judged by SDS–PAGE) which co-purifies and co-immunoprecipitates with Golgi apparatus casein kinase (G-CK) from rat lactating mammary gland has been shown by mass spectrometric sequence analysis to be identical or very similar to the glucose-regulated protein, GRP94. GRP94 is also readily phosphorylated by G-CK (K_m = 0.2 μM) at seryl sites which are different from the sites affected by casein kinase-2 (CK2) in the same protein. A study with peptide substrates would indicate that the G-CK sites in GRP94 conform to the motif S-R/K-E-X (X being different from D and E) which is not recognized by CK2.

Key words: Casein kinase; Protein kinase; Golgi apparatus; GRP94; Endoplasmin; Phosphorylation

1. Introduction

Most Ser/Thr protein kinases recognize sites specified by basic and/or prolyl residues. In contrast, few of them are acidophilic in nature in that their consensus sequences rely on carboxylic and/or previously phosphorylated side chains acting as specificity determinants [1]. Often, these acidophilic protein kinases are referred to with the conventional term 'casein kinases' as they share the ability to phosphorylate casein in vitro. In some instances, this feature does not reflect any physiological role in casein phosphorylation: thus type 1 and type 2 'casein kinases' (recently re-named as protein kinases CK1 and CK2) are ubiquitous and pleiotropic enzymes totally unrelated to casein [2–5].

In contrast, bona fide casein kinase activity is present in the Golgi apparatus of lactating mammary gland, where it is committed to the phosphorylation of casein fractions prior to their secretion with milk. Despite the fact that this Golgi apparatus casein kinase (G-CK, also previously termed GEF-CK, 'Golgi-enriched fraction casein kinase') has been known for a long time (e.g. [6,7]), its primary structure is still undeciphered. Little information is available about its biochemical properties, except for its site specificity which has been thoroughly investigated using casein fractions and synthetic peptides as phosphorylatable substrates [8–11]. These studies showed that the G-CK consensus is crucially dependent on a glutamic acid (or a previously phosphorylated serine) at position *n*+2 relative to the target amino acid, generating the motif S-X-E/Sp, which is similar to, but definitely distinct from the minimum consensus of CK2, S/T-X-X-E/D/Sp/Yp [11,12].

Based on these differences in specificity, a highly selective peptide substrate has been developed [11], which allowed the detection of G-CK not only in mammary gland, but also in the Golgi apparatus of a variety of tissues [13] where its casein kinase activity would otherwise be masked by predominant activity of CK1 and CK2. This finding raised the possibility that several proteins already known to contain phosphorylated S-X-E motifs in sequences not suited for other classes of ubiquitous 'casein kinases', with special reference to highly pleiotropic CK2, might be natural substrates of G-CK. This of course would especially apply to proteins either secreted from the cell or anyway functionally connected to the Golgi apparatus, where G-CK appears to be specifically located [13]. The list of such putative substrates of G-CK is quite long, including, among others, fibrinogen, ovalbumin, pepsinogen, ACTH, furin, osteopontin, matrix Gla protein and mannose-6-P receptor (see [13,14] and references therein), but only sporadically, notably in the case of osteopontin [15], was the ability of G-CK to catalyze the postulated phosphorylation demonstrated.

Here, we show that, in an attempt to purify G-CK to homogeneity from rat lactating mammary gland, a phosphoprotein co-elutes with the kinase activity throughout all the purification steps. This protein turns out to be very similar if not identical to glucose-regulated protein-94 (GRP94), belonging to the stress protein family, and to undergo phosphorylation by G-CK at sites different from those affected by CK2.

2. Materials and methods

2.1. Chemicals, biochemicals and peptides

[γ-^{32}P]ATP, enhanced chemiluminescence detection system and secondary antibodies were purchased from Amersham (Arlington Heights, USA), anti-GRP94 antibodies were from Stress-Gen Biotechnologies Corp. (Victoria, B.C., Canada) and the other chemicals were from Sigma Chemical Company (Dorset, UK). Anti-*p*-fluorosulfonylbenzoyl 5′-adenosine (FSBA) antibodies were kindly provided by Dr. P.J. Parker (London, UK). GRP94-derived peptides were synthesized by solid phase peptide synthesis [16] on a 4-hydroxymethyl-phenoxymethylcopolystirene–1% divinylbenzene resin (Perkin Elmer) using a peptide synthesizer (Model 431-A, Applied Biosystems) according to the manufacturer's protocol.

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Abbreviations: G-CK, Golgi apparatus casein kinase; CK2, casein kinase-2; GRP, glucose-regulated protein; FSBA, *p*-fluorosulfonylbenzoyl 5′-adenosine

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The purity of crude peptides was 85% or more on the basis of peak areas as determined by analytical HPLC. The molecular weights checked with a KRATOS MALDI-TOF MS (Shimadzu) agreed well with the theoretical values.

2.2. Protein kinases

G-CK was routinely purified from the lactating mammary gland of 10 rats. The Golgi apparatus was isolated as detailed elsewhere [13,17].

The purity of the Golgi fraction was documented by its high galactosyl transferase activity (≥270 nmol/h/mg) as opposed to undetectable NADH–cytochrome *c* reductase and glucose-6-phosphatase activities. Extraction of G-CK from the particulate fraction was performed as described previously [13]. The soluble extract was subjected to three purification steps as outlined in Fig. 1A. The chromatography through DEAE-Sepharose and Heparin-Sepharose columns was performed as described previously [13]. The peak of G-CK activity, eluted from Heparin-Sepharose at 0.5 M NaCl, was then chromatographed through a FPLC-Superdex 200 column (10 mm × 300 mm), equilibrated with buffer A (20 mM Tris–HCl pH 7.5, 5 mM magnesium acetate, 1 mM EGTA, 10 mM 2-mercaptoethanol, 0.1% Triton X-100, 10% glycerol, 0.05 mM phenylmethylsulfonyl fluoride (PMSF), 0.02% NaN_3) containing 500 mM NaCl. 0.2 ml fractions were collected at a flow rate of 0.4 ml/min. At this stage, G-CK, eluted as a broad peak of activity displaying an apparent M_r of 400 kDa (Fig. 1B), was still heavily contaminated by a 94 kDa band subsequently identified as GRP94 (see inset Fig. 1B). To remove GRP94, G-CK was passed through a ConA-Sepharose column (5 ml), equilibrated with buffer B (20 mM Tris–HCl pH 7.5, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 10 mM 2-mercaptoethanol, 10% glycerol, 0.05 mM PMSF); G-CK activity was eluted in the flowthrough, while GRP94 was retained on the column and subsequently eluted with buffer B containing 0.6 M α-D-methylmannoside.

Purification of rat liver CK2 was performed essentially as described elsewhere [18].

2.3. Reaction with FSBA

The G-CK fraction eluted from DEAE-Sepharose (10 μg) was incubated either alone or with 0.5 mM FSBA and 10 mM $MnCl_2$, in the absence or presence of 2 mM ATP at 30°C for 1 h [19]. Reacted proteins were resolved by 8% SDS–PAGE, transferred onto nitrocellulose and immunostained with anti-FSBA antibody at a dilution of 1:500.

2.4. In-gel phosphorylation assay

G-CK from DEAE-Sepharose (50 μg) was subjected to SDS–PAGE followed by in situ denaturation/renaturation [20]. After renaturation, the membrane was incubated for 30 min at 30°C in a buffer containing 50 mM Tris–HCl, 10 mM $MnCl_2$, 50 μM [γ-^{32}P]ATP (3000–6000 Ci/mmol). The membrane was washed twice for 10 min in 30 mM Tris–HCl (pH 7.5), once in 0.1% NP-40 and once more in 30 mM Tris–HCl (pH 7.5). The radioactivity incorporated was detected by Instant Imager (Packard).

2.5. Microsequencing

15 μg of the G-CK containing fraction, eluted from Superdex 200, was subjected to SDS–PAGE and 170 and 94 kDa bands were identified by Coomassie staining. The two bands were then excised, destained and tryptically digested as described elsewhere [21]. The solution containing the tryptic fragments was then electrosprayed into a Finnigan LCQ ion trap. Peptides of interest were isolated by trapping and then fragmented. The ions were labeled according to Roepstorff [22]. The resulting MS/MS spectra were either manually interpreted or used for database searching using the Sequest program. The MS/MS spectrum of one of the peptides is shown in Fig. 3 together with its interpretation. The rest of the sequences are indicated in Section 3.

2.6. Purification of GRP94 from rat liver microsomes

The microsomal fraction was extracted, as previously described [13], and subjected to a DEAE-Sepharose followed by a Heparin-Sepharose column as previously described [13]. The eluted fractions were collected and assayed for immunoreactivity against anti-GRP94. The GRP94 peak, eluted from Heparin-Sepharose at 0.5 M NaCl, was chromatographed on FPLC-Superdex 200 (10 mm × 300 mm), previously equilibrated with buffer A containing 500 mM NaCl. 0.2 ml fractions were collected at a flow rate of 0.4 ml/min. The immunoreactive peak with an apparent M_r of 200 kDa was passed through a ConA-Sepharose column (5 ml), equilibrated with buffer B. GRP94 was eluted with buffer B containing 0.6 M α-D-methylmannoside. The final preparation was free of any kind of 'casein kinase' activity as also judged by using peptide substrates specific for CK1, CK2 and G-CK.

2.7. Phosphorylation assays

Phosphorylation of GRP94 (250 nM) by G-CK (50 ng) was performed at 30°C in 40 μl of an incubation mixture containing 50 mM Tris–HCl pH 7.5, 10 mM $MnCl_2$, 50 μM [γ-^{32}P]ATP (specific activity 1500 cpm/pmol) and 0.1 μM polylysine. When the phosphorylating kinase was CK2 (200 ng), $MnCl_2$ was replaced by 10 mM $MgCl_2$ and 150 mM NaCl was also added to the incubation medium. At the end of incubation (10 min), the samples were subjected to 8% SDS–PAGE and the radioactive bands were detected and quantified by means of Instant Imager (Packard). Phosphorylation of peptides and kinetic analyses were performed as in [13], except for the omission of $MgCl_2$ from the incubation medium. For routine assay of G-CK, the specific β-casein-derived peptide substrate β(28–40) [11] was used as phosphate acceptor.

2.8. Immunoprecipitation

Either Golgi fraction (200 μg) or G-CK purified after ConA-Sepharose treatment (5 μg) or GRP94 (1 μg) were pre-cleared by incubation with protein A-Sepharose for 45 min at 4°C. Subsequently, the samples were incubated overnight at 4°C with anti-GRP94. The immunocomplexes were analyzed for G-CK activity by incubation with the specific peptide substrate β(28–40) and [γ-^{32}P]ATP as described above, and for the presence of GRP94 by Western blotting using anti-antibody at a 1:500 dilution.

2.9. Phosphoamino acid analysis

GRP94, phosphoradiolabeled after incubation with either G-CK or CK2 and [γ-^{32}P]ATP, was resolved by SDS–PAGE. The protein was electrophoretically transferred onto a nitrocellulose filter and radiolabeled GRP94 was localized by autoradiography. The excised band was tryptically digested and then hydrolyzed with 6 M HCl hydrolysis at 110°C for 4 h. Radiolabeled phosphoamino acids were isolated by pH 1.9 high-voltage paper electrophoresis and evidenced by autoradiography [23].

3. Results

A summary of the purification steps of G-CK is provided in Fig. 1A. The activity was monitored by using the specific peptide substrate, KKKIEKFQSEEQQQ, which is not affected by other 'casein kinases' [13]. During the final purification step on Superdex 200, G-CK activity was eluted as a broad symmetric peak of apparent M_r around 400 kDa (Fig. 1B). As shown in the inset of Fig. 1B, the profile of G-CK activity was paralleled by the appearance of two phosphoradiolabeled bands of about 170 and 94 kDa, respectively, if the eluted fractions were incubated with [γ-^{32}P]ATP and Mg^{2+}. Both bands were also detectable by anti-FSBA immunostaining but only the 170 kDa band was detectable by in-gel autophosphorylation, suggesting that it might be the kinase itself (Fig. 2). Attempts to obtain sequence information from it failed due to very low fragmentation yield; one fragment however, GDXXXXDFGXXR, displayed the highly conserved DFG motif found in protein kinase domain VII.

In contrast, the 94 kDa band yielded five tryptic fragments, which were analyzed by MS/MS spectra (one of which is shown as an example in Fig. 3). All of the sequences obtained (EEEAIQLDGLNASQIR; GTTITLVKEEASDYLELDTIK; EFEPLLNWMK; SILFVPTSAPR; VFITDDFHDMMPK) matched that of mouse GRP94.

It can therefore be concluded that the 94 kDa phosphory-

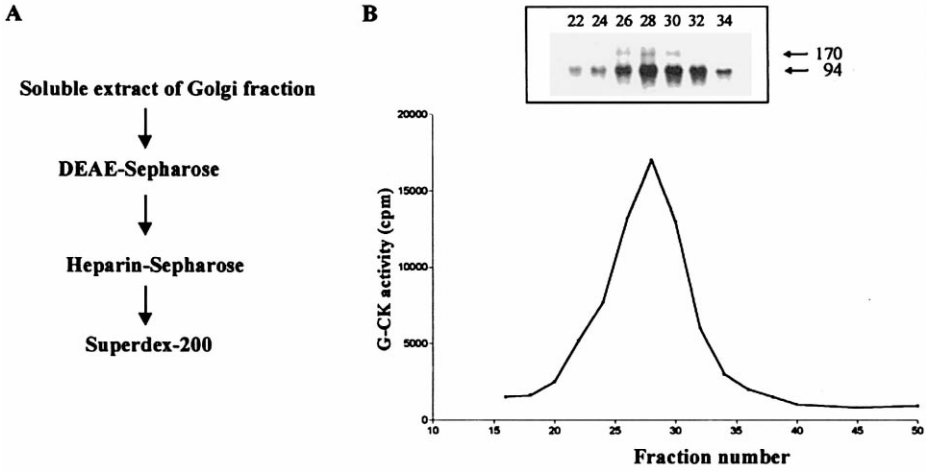


Fig. 1. Purification of G-CK. A synopsis of the purification procedure of G-CK from soluble extract of Golgi-enriched fraction is shown in A. B shows the profile of G-CK activity eluted from Superdex 200. The inset refers to the autophosphorylation of individual fractions (as indicated) subjected to SDS–PAGE and autoradiography. The experimental conditions for gel filtration, protein kinase assay and autophosphorylation are detailed in Section 2.

latable protein associated with G-CK throughout all the purification steps is GRP94 or a very closely related protein. Tight interaction between G-CK and GRP94 has been confirmed by immunoprecipitating GRP94 from the extract of mammary gland Golgi apparatus with anti-GRP94 antibodies and by showing that significant amounts of G-CK activity, as judged from the specific peptide phosphorylation, are present in the immunoprecipitate (Fig. 4).

Once established that GRP94 is associated with G-CK, we wanted to assess if its phosphorylation was actually mediated by G-CK itself. GRP94 is known to be a substrate of another 'casein kinase', CK2 [24], which however is hardly detectable in the Golgi apparatus [13]. To compare the abilities of G-CK and CK2 to phosphorylate GRP94, this latter was purified free of G-CK from rat liver microsomes and subjected to in vitro phosphorylation assays in the presence of either G-CK (rendered free of GRP94 by ConA-Sepharose treatment as described in Section 2.2) or CK2. As shown in Fig. 5, no significant autophosphorylation of GRP94 could be detected, at variance with a previous report [25]. Instead, GRP94 was readily phosphorylated by either G-CK or CK2 (Fig. 5). The phosphorylation stoichiometry approached, with both kinases, 1 mol P/mol (not shown); however, the kinetic constants and, even more, the SerP/ThrP ratio were quite different. GRP94 displayed higher affinity for G-CK as compared to CK2 (K_m values 0.20 vs. 0.50 μM), and the latter preferentially phosphorylates threonyl residues, whereas only seryl residues were affected by G-CK (see Fig. 5), in agreement with

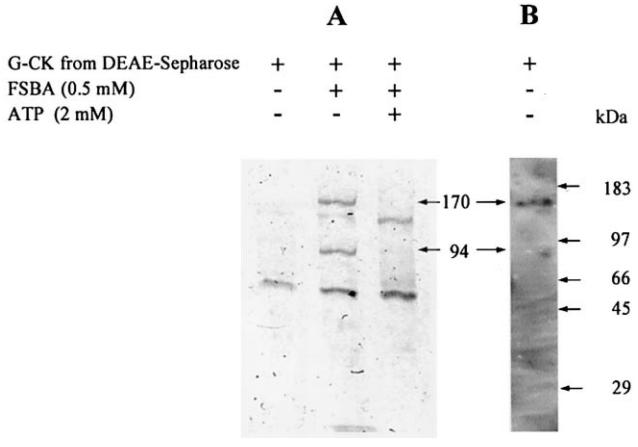
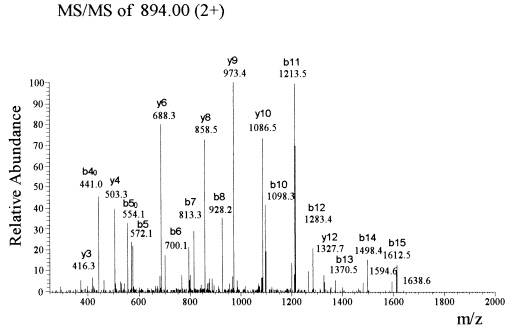


Fig. 2. Anti-FSBA immunoreactivity and in-gel autophosphorylation of G-CK. The experiments were performed using 30 μg of G-CK preparation eluted from DEAE-Sepharose. A: anti-FSBA immunoreactivity. Lane 1, G-CK alone; lanes 2 and 3, G-CK in the presence of FSBA (0.5 mM) either alone or in the presence of 2 mM ATP. B: autoradiography of phosphorylation assay following in situ denaturation/renaturation of G-CK (see Section 2).



FRAGMENT ION MASSES:

a-d n	a	b	y	v-z n
0	---	1.01	1785.90	16
1	102.06	130.05	1656.86	15
2	231.10	259.09	1527.81	14
3	360.14	388.14	1398.77	13
4	431.18	459.17	1327.73	12
5	544.26	572.26	1214.65	11
6	672.32	700.32	1086.59	10
7	785.40	813.40	973.51	9
8	900.43	928.43	858.48	8
9	957.45	985.45	801.46	7
10	1070.54	1098.53	688.37	6
11	1184.58	1212.57	574.33	5
12	1255.62	1283.61	503.29	4
13	1342.65	1370.64	416.26	3
14	1470.71	1498.70	288.20	2
15	1583.79	1611.79	175.12	1
16	1739.89	1767.89	19.02	0

Fig. 3. MS/MS analysis of tryptic digestion of GRP94. The figure shows the MS/MS spectrum obtained from 2+ peptide ion *m/z* 894.0 in the ion trap mass spectrometer. For details, see Section 2.

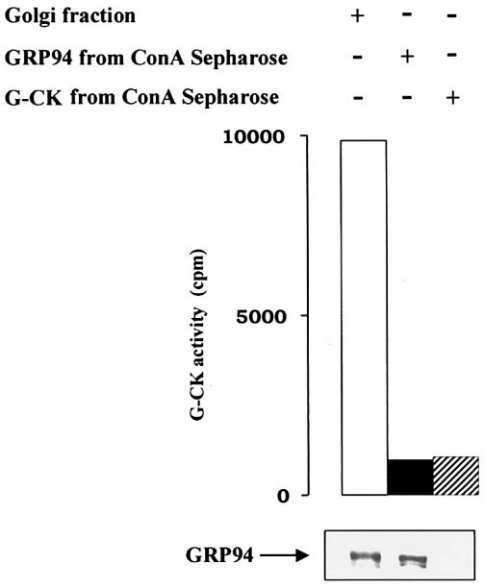


Fig. 4. Co-immunoprecipitation of G-CK with GRP94 from Golgi extracts. Anti-GRP94 immunoprecipitates from different sources (as indicated) were subjected to in vitro kinase assays by the specific β(28–40) peptide substrate (histogram) and analyzed for the presence of GPR94 by Western blotting (inset). Experimental conditions are detailed in Section 2.

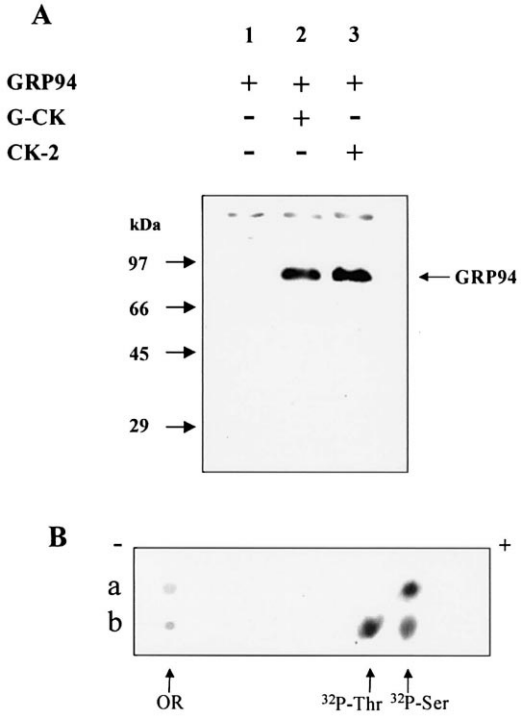


Fig. 5. Phosphorylation of GRP94 by G-CK and CK2. The autoradiography after SDS–PAGE is shown in A. The molecular weight markers (kDa) are indicated on the left side. In B, the GRP94 radiolabeled bands detected in lanes 3 (a) and 5 (b) of A were blotted to nitrocellulose, excised and eluted by tryptic digestion, followed by 6 N HCl hydrolysis as described in Section 2. ^{32}P-serine and ^{32}P-threonine were isolated by high-voltage paper electrophoresis at pH 1.9 (see Section 2) and detected by autoradiography.

the notion that threonine is not a good target for G-CK [1,11]. The phosphorylation of threonyl residues by CK2 did not come as a surprise either, since the main radiolabeled peptide from GRP94 phosphorylated by CK2 corresponded to a C-terminal segment of GRP94 including four threonines, all displaying the CK2 consensus sequence [24]. Five consensus sequences for G-CK (S-X-E) are found in GRP94, namely S-17, S-326, S-342, S-426 and S-586. Curiously, all these potential sites conform to the motif S-R/K-E, with a basic residue inserted between the phosphoacceptor residue and the acidic determinant at position *n*+2. This feature would be detrimental to CK2 phosphorylation [1], while it is well tolerated by G-CK (unpublished data). All five tridecapeptides reproducing the putative G-CK sites were phosphorylated by G-CK albeit with different efficiencies (Table 1); in contrast, none of them was phosphorylated by CK2 to any appreciable extent (not shown). It is quite likely therefore that one or more of these residues are also the one(s) which are phosphorylated by G-CK in full length GRP94.

4. Discussion

The work described here adds a new member to the still short but growing list of substrates of the G-CK. For a long time, G-CK was believed to be a highly dedicated kinase, specifically committed just to the phosphorylation of casein fractions within lactating mammary gland. Recently however, using a highly selective peptide substrate instead of casein, G-CK was also detected in the Golgi apparatus of a variety of tissues where casein is not present [13], disclosing the possibility that a number of phosphoproteins known to contain the phosphorylated motif Sp-X-E, identical to the one recognized by G-CK, might be targets of this enzyme. While the list of these putative G-CK substrates is quite long, including among

Table 1
Kinetic constants for the phosphorylation of GRP94-derived synthetic peptides by G-CK

Nomenclature	Sequence	G-CK		
		K_m (mM)	V_{max} (nmol/min/mg)	V_{max}/K_m
GRP94 (12–24)	EDLGKS[17]REGARAP	0.220	0.37	1.6
GRP94 (320–333)	KWQRPS[326]KEVEEDE	0.125	2.97	23.7
GRP94 (337–349)	KYKAFS[346]KEADDPM	1.659	18.60	11.2
GRP94 (420–433)	KPLNVS[426]RETLQQH	1.659	15.00	9.0
GRP94 (580–593)	KKTKES[586]REATEKE	0.086	3.3	38.2

Experimental conditions are described in Section 2 with the exception that peptide concentrations were varied. Values are means of at least three determinations with a standard error less than 15%. Phosphorylatable serines are numbered according to dog GRP94. Underlining denotes the glutamic acid supposed to act as specificity determinant.

others pepsin, fibrinogen, ACTH, progastrin, furin, osteopontin, matrix Gla protein, mannose-6-P receptor (see [13,14] and references therein), only a in few cases, their actual phosphorylation by G-CK has been documented, namely with osteopontin [15] and the salivary APRP [26].

Here, we show that another bona fide substrate of G-CK is the Ca^{2+} binding GRP94, also termed endoplasmin. This protein belongs to the stress protein family, whose synthesis is enhanced when extracellular glucose is low, intracellular Ca^{2+} stores are depleted or glycosylation is inhibited [27]. GRP94 is 50% homologous to 90 kDa heat shock protein (HSP90). Both GRP94 and HSP90 have been reported to undergo significant autophosphorylation [25,28] as well as phosphorylation by protein kinase CK2 [24,29]. In our hands, no autophosphorylation of GRP94 could be detected using a highly purified preparation from rat liver microsomes entirely free of casein kinase activity (Section 2.6); the same preparation is readily phosphorylated by either CK2 or G-CK at residues which are partially if not entirely different. Based on our data, the first choice candidates for undergoing phosphorylation by G-CK are sites displaying the sequence S-R/K-E, a motif which is also found in HSP90 and which is refractory to CK2 phosphorylation. It would be interesting to check whether other proteins containing this motif are also susceptible to G-CK phosphorylation.

The physiological relevance of G-CK-catalyzed GRP94 phosphorylation is strongly supported by its low K_m value (0.2 μM) and by the finding that the two proteins interact with each other, being co-immunoprecipitated from extracts of lactating mammary gland Golgi apparatus and undergoing co-elution during several steps of the G-CK purification procedure. The detection of substantial amounts of Golgi-associated GRP94 was rather unexpected considering the major localization of this protein to the ER fraction, by virtue of its C-terminal ER retention KDEL motif. The possibility that our finding might be accounted for by contaminations from ER membranes is hardly conceivable, considering the high degree of purification of the Golgi preparation used in this study as judged from its lack of detectable activity of marker enzymes of particulate fractions other than the Golgi apparatus itself (see Section 2.2). On the other hand, the detection of GRP94 exported from the ER to other subcellular fractions, including the Golgi apparatus, has already been reported (e.g. [30–32]). In this respect, it is tempting to speculate that binding to G-CK is the driving force contributing to the Golgi localization of GRP94. Pertinent to this might also be the observation that lactating mammary gland is the tissue where by far the highest expression of G-CK has been found [13]. It would be interesting therefore to check in different tissues whether there is a correlation between G-CK expression and Golgi localization of GRP94. The molecular features of the interaction between G-CK and GRP94 are presently unknown. Considering however that GRP94 tends to dimerize [33] and that the apparent M_r of G-CK/GRP94 complex eluted from Superdex 200 approximates 400 kDa, it seems likely that one GRP94 dimer binds to one G-CK molecule, assuming for this latter a M_r around 170 kDa (see Fig. 2). At present, it is not clear whether the formation of these supramolecular complexes is a mere consequence of enzyme–substrate interaction or it reflects more complicated functional relationships between GRP94 and G-CK. However, the much higher affinity for G-CK of full size GRP94 as compared to its phosphoacceptor peptides, as judged from three orders of magnitude lower K_m values, would be more consistent with the latter hypothesis.

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