Golgi apparatus mammary gland casein kinase: monitoring by a specific peptide substrate and definition of specificity determinants

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Abstract The casein kinase from the Golgi apparatus of lactating mammary gland (GEF-CK) is distinct from ubiquitous 'casein kinases' termed protein kinases CK1 and CK2 and appears to define a family of secretory pathways protein kinases that phosphorylate seryl residues followed by an acidic residue at position +2. In this report we show that a new synthetic peptide substrate derived from β-casein (β[28–40]) is suitable for the fast, efficient and selective monitoring of GEF-CK, being unaffected by CK1 and CK2, and we define the consensus sequence of this protein kinase as being Ser-Xaa-Glu/SerP, distinct from that of CK2 (Ser/Thr-X-X-Glu/Asp/SerP/TyrP). In particular, the failure to recognize Asp as crucial specificity determinant prevents the phosphorylation of the specific CK2 peptide substrate RRRADDSDDDDD by GEF-CK. Thus, peptide substrates are now available for the fast and specific monitoring of all the three classes of 'casein kinases', CK1, CK2 and GEF-CK.

Key words: Casein kinase; Protein kinase CK1; Protein kinase CK2; Mammary gland; Golgi apparatus

1. Introduction

Most Ser/Thr protein kinases recognize consensus sequences specified by basic residues situated in the proximity of the target amino acid. A minority of Ser/Thr protein kinases however use acidic residues as specificity determinants [1]. Symptomatic of such an acidophilic nature often is the attitude to phosphorylate casein in vitro, whence the operational term 'casein kinase' commonly applied to three different kinds of acidophilic Ser/Thr protein kinases [2]. Two of these, however, formerly termed casein kinases 1 and 2, are ubiquitous and pleiotropic protein kinases, functionally unrelated with casein and thereafter recently re-termed protein kinases CK1 and CK2 (reviewed in [3]). A third class of casein kinases includes the enzyme(s) committed with the biosynthetic phosphorylation of casein and mainly localized in the Golgi apparatus of the mammary gland [4,5]. In contrast to CK2 and CK1, these genuine casein kinase(s) have been only poorly characterized, mostly using crude extracts of mammary gland particulate fractions. A genuine casein kinase termed 'Golgi-enriched fraction casein kinase' (GEF-CK) has been isolated from guinea-pig mammary gland and partially purified as an about 70 kDa enzyme [6,7]. As expected GEF-CK was able to phosphorylate dephosphorylated casein at the same sites which are phosphorylated in native casein. From both the inspection of these sites [8] and studies with synthetic peptide substrates [9,10] it was concluded that the consensus sequence of GEF-CK is S-X-acidic, which is distinct from but similar to that of CK2 (S/T-X-X-acidic). Consequently the best peptide substrates for CK2, including multiple acidic residues downstream from serine are also readily phosphorylated by GEF-CK [9]. In contrast, it has been observed that several phosphorylated sites present in naturally occurring phosphoproteins, e.g. pepsin, ovoalbumin, ACTH, fibrinogen, exhibit the minimal S-X-acidic-X sequence which is not suitable for CK2 while it could be phosphorylated by a protein kinase having the same site specificity as GEF-CK [2]. Indeed, the phosphorylation of previously dephosphorylated pepsin by a crude preparation of bovine mammary gland casein kinase has been reported [4].

These observations prompted us to start a thorough characterization of GEF-CK, having the ultimate goal of unraveling its primary structure and assessing the presence of related enzymes in tissues other than the mammary gland.

Here we report the design of a peptide substrate suitable for the selective detection and monitoring of GEF-CK and protein kinase(s) sharing the same consensus sequence and we disclose additional features that differentiate the specificity of GEF-CK from those of CK2 and CK1.

2. Materials and methods

2.1. 'Casein kinases'

Isolation and partial purification of a membrane-bound casein kinase from the Golgi-enriched fraction of rat lactating mammary gland was performed as in [7] using ATP-agarose column chromatography. Rat liver protein kinases CK1 and CK2 were purified as previously described [11].

2.2. Synthetic peptides

The peptides KKIEKFQSEEQQQ, KKIEKFQSAEAAA, RRRAEESEEEEE, RRRAEESEAEEE, RRRADDSDEDDD were prepared in solid phase peptide synthesis by Fmoc (9-fluorenylmethoxycarbonyl) chemistry, using an automatic synthesizer (model 431 A, Applied Biosystems) at 0.05 mmol scale as previously described [12]. The peptides RRRAEESEDEEE and SSSDD were synthesized by a manual procedure as described in [13] using Fmoc solid-phase chemistry on a polystyrene support (Wang resin) preloaded with the first amino acid (0.9 mmol/g). After the purification of the peptides, the purity, checked by amino acid analysis and analytical HPLC, was ≥95%. The synthesis of the peptide SSSEE, SSSSpSp, SSSTpTp and SSSYpYp is described elsewhere [14]. The peptides RRREEESDDD and RRRDDDSEEE were kindly provided by Dr. O.G. Issinger (Homburg).

2.3. Phosphorylation assays

GEF-CK (0.8–1.2 μg) was incubated for 20 min at 30°C in a medium containing 80 mM MOPS pH 6.3, 15 mM $MgCl_2$, 15 mM $MnCl_2$, 100 μM [γ-^{32}P]ATP (spec. act. 500 cpm/pmol) in the presence of either casein or peptide substrates. The concentration of casein was 1 mg/ml and that of peptide substrates was 1 mM, unless differently indicated. Incorporation of ^{32}P was evaluated by either the phosphocellulose paper procedure [15] or following partial acid hydrolysis of the phosphorylated peptide and isolation of radioactive phosphoamino acids as described in [16]. CK1 and CK2 were assayed as previously described [12]. Kinetic constants of peptide substrates were determined

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Table 1
Selective phosphorylation of peptide substrates by three distinct 'casein kinases'

		Phosphorylation rate (pmol·min^{-1}) by		
		GEF-CK	CK2	CK1
I	KKIEKFQSEEQQQ	3.81	0.01	0.02
II	KKIEKFQSAEAAA	1.85	<0.01	<0.01
III	RRRADDSDDDDD	0.32	2.03	0.04
IV	RRKDLHDDEEDEAMSITA	0.43	0.13	4.52
	Casein	1.00	1.00	1.00

All peptide substrates were 1 mM. The amounts of GEF-CK, CK2 and CK1 were equivalent to one unit of casein kinase activity, corresponding to 1 pmol P transferred from ATP to casein per min. Experimental conditions are either detailed or referenced in section 2, except for ATP concentration that was 100 μM also with CK2 and CK1. Peptide I corresponds to the 28–40 sequence of β-casein [17]. Peptide II is a variant of peptide I in which the modifications are underlined. Peptides III and IV are optimal model substrates for CK2 and CK1, respectively [12].

by regression analysis of double-reciprocal plots constructed from initial rate measurements.

3. Results

Searching for a synthetic peptide substrate specific for GEF-CK, the sequence around Ser-35 of β-casein was chosen because this residue is fully phosphorylated in native β-casein [17] and, unlike other phosphorylated sites of casein, it displays the canonical consensus for GEF-CK (S-X-E) but not that for CK2 (S-X-X-E/D). This peptide would be expected therefore to be phosphorylated by GEF-CK alone, by contrast to the other phosphoseryl site of β-casein (Ser 17–19) which is an excellent phosphoacceptor substrate for CK2 as well [10]. The natural sequence of the β[28–40] peptide moreover includes 3 lysyl residues suitable for the fast and simple phosphocellulose assay [15]. A derivative of the β[28–40] peptide was also prepared in which glutamic acid at position +1, a positive, albeit not sufficient determinant for phosphorylation by CK2 [18], was replaced by alanine, with the aim to reduce further the probability of phosphorylation by CK2.

The phosphorylation of these peptides by equivalent amounts (in terms of casein kinase units) of GEF-CK, CK2 and CK1 are reported in Table 1, where they are compared with the specific peptide substrates for CK2 and CK1. It can be seen that the β[28–40] peptide is readily phosphorylated by GEF-CK alone, both CK2 and CK1 being practically ineffective on it. Such an outcome makes the replacement of glutamic acid at position +1 unnecessary for improving specificity (compare peptides I and II in Table 1).

Table 1 also shows that GEF-CK is nearly inactive on either the CK2 or the CK1 peptide substrates. While its incapability to phosphorylate the CK1 peptide was expectable, its failure to phosphorylate the CK2 peptide came as a surprise considering that this peptide includes an aspartic acid at position +2 and that other peptides with similar acidic clusters downstream from serine, like SEEEEE, are excellent substrates for both CK2 and GEF-CK [9].

A possible explanation for the failure of GEF-CK to phosphorylate the RRRADDSDDDDD peptide substrate of CK2 could be that aspartic acid cannot replace glutamic acid in the consensus sequence recognized by GEF-CK. To check this hypothesis a series of derivatives of the RRRADDSDDDDD peptide in which aspartyl residues were variably replaced by glutamyl ones were assayed as substrates for GEF-CK (Table 2). It can be seen that indeed the replacement of the individual aspartic acid at position +2 with glutamic acid in the peptide RRRADDSDDDDD (to give RRRADSDEDDD) is sufficient to convert a nearly inert substrate into the best substrate among those of Table 2. In a specular way the phosphorylation of the excellent peptide substrate RRRAEESEEEEE is compromised by the individual replacement of Glu by Asp at position +2, almost as severely as by substituting Glu by Ala. Additional evidence that glutamic acid but not aspartic acid can act as crucial specificity determinant with GEF-CK is provided by comparing the peptides RRRDDDSEEE and SSSEE (both good substrates) with RRRDDDSDDD and SSSDD, respectively, both of which are almost unaffected.

The failure of aspartic acid to surrogate glutamic acid in the consensus sequence for GEF-CK prompted us to assess the efficacy of phosphorylated residues as well, two of which, namely TyrP and SerP are known to act as powerful specificity determinants with CK2 [14]. The data of Table 2 clearly show that neither phosphotyrosine nor phosphothreonine can surrogate glutamic acid in peptides exhibiting the consensus sequence for GEF-CK (compare peptide 11 with peptides 14 and 15), while phosphoserine does act as specificity determinant, less effectively however than glutamic acid (compare peptide 11 with peptide 13).

The kinetic constants of GEF-CK for its peptide substrates

Table 2
Specificity determinants of GEF-CK: effect of substituting glutamic acid by other residues

		Phosphorylation rate (pmol·min^{-1}·mg^{-1})
1	KKIEKFQSEEQQQ	1433
2	KKIEKFQSAEAAA	885
3	RRRAEESEEEEE	1632
4	RRRAEESEDEEE	418
5	RRRAEESEAEEE	169
6	RRRADDSDDDDD	193
7	RRRADDSDEDDD	2346
8	RRRDDDSDDD	103
9	RRREEESDDD	99
10	RRRDDDSEEE	1722
11	SSSEE	1303
12	SSSDD	207
13	SSSSpSp	436
14	SSSTpTp	n.d.[a]
15	SSSYpYp	n.d.[a]

The concentration of all peptides was 1 mM. Experimental conditions are detailed in section 2. Peptide 1 is the β[28–40] peptide corresponding to the 28–40 sequence of β-casein. Peptides 3–7 are variants of the peptide RRRAEESEEEEE, changes from which are underlined.
[a]Not detectable.

Table 3
Kinetic constants of peptide substrates for GEF-CK (A) and CK2 (B)

		K_m (μM)	V^a_{max}	V_{max}/K_m
(A) GEF-CK				
1	KKIEKFQSEEQQQ	663	2747	4.14
2	KKIEKFQSAEAAA	785	1743	2.22
3	RRRAEESEEEEE	15	2350	156.66
4	RRRAEESEDEEE	1720	1198	0.69
5	RRRAEESEAEEE	2805	654	0.23
6	RRRADDSDDDDD	3062	454	0.14
7	RRRADDSDEDDD	65	2571	39.55
8	RRRDDDSDDD	2218	224	0.10
9	RRREEESDDD	1438	112	0.07
10	RRRDDDSEEE	43	1783	41.46
11	SSSEE	285	2164	7.59
12	SSSDD	n.m.[b]	n.m.[b]	n.m.[b]
13	SSSSpSp	657	1114	1.69
(B) CK2				
3	RRRAEESEEEEE	23	74	3.21
4	RRRAEESEDEEE	13	149	11.46
5	RRRAEESEAEEE	19	86	4.52
6	RRRADDSDDDDD	13	116	8.92
7	RRADDSDEDDD	19	137	7.21

Kinetic experiments were performed as described in section 2. Peptides 3–7 are variants of the peptide RRRAEESEEEEE, changes from which are underlined. Values are means of at least three determinations with a standard error less than 15%.
[a]V_{max} values are expressed either in pmol min^{-1} mg^{-1} (A) or nmol min^{-1} mg^{-1} (B).
[b]Not measurable due to too low a phosphorylation rate.

are reported in Table 3A. They show that the beneficial effect of glutamic acid at position +2 as compared to aspartic acid is largely accounted for by differences in K_m values, increasing, e.g. from 15 to 1720 μM by just replacing Asp for Glu at position +2 in the peptide RRRAEESEEEEE (to give RRRAEESEDEEE), and conversely dropping from 3,062 to 65 μM if Asp is replaced by Glu at position +2 in the peptide RRRADDSDDDDD (to give RRRADDSDEDDD). In this latter case also the V_{max} is greatly altered, increasing from 454 to 2,571 pmol min^{-1} mg^{-1}. A similar change of both K_m and V_{max} is found between the related peptides RRRDDDSEEE and RRRDDDSDDD, the overall phosphorylation efficiency of the latter resulting 500-fold lower than that of the former peptide.

Interestingly the behaviour of CK2 is opposite under this respect since it displays a preference for an aspartic over a glutamic acid at position +2 (see Table 3B). The kinetic constants of Table 3B also corroborate the concept that position +2 is relatively unimportant for substrate recognition by CK2 since even a neutral residue in that position is not harmful, giving rise to a derivative (RRRAEESEAEEE) which is phosphorylated slightly better than the parent peptide with a glutamic acid there, though somewhat less efficiently than the derivative with Asp instead of Ala. This further differentiates CK2 from GEF-CK, whose requirement for a glutamic acid (or phosphoserine) at position +2 is absolute [9].

The kinetic constants of Table 3A also show that the specific peptide substrate β[28–40] exhibits the highest V_{max} value but a relatively high K_m value (663 μM) as compared to more acidic peptides, corroborating the concept [9] that multiple N terminal acidic residues in addition to that at position +2, though not required for optimal phosphorylation rate, increase the affinity for the enzyme. In this respect the low K_m value of the peptide RRRADDSDEDDD is noteworthy since in it the acidic residues at positions other than +2 are those of aspartic acid, i.e. an amino acid which at position +2 fails to act as specificity determinant (see above). It should be concluded therefore that while the consensus sequence for GEF-CK is S-X-E/Sp, excluding aspartic acid as specificity determinant at position +2, aspartyl residues may nevertheless play a favourable role on K_m if present at different positions.

4. Discussion

Despite the fact that casein was the first phosphorylated protein to be detected, over one century ago [22] and therefore casein kinase(s) were the first protein kinases whose existence was implicitly recognized, very little is known about these enzymes. Their primary structure is still obscure in sharp contrast with the detailed knowledge we already have of the structure of more than 300 younger members of the protein kinase superfamily [23] and their catalytic and regulatory properties are very poorly understood. This lack of information is especially regrettable considering that it is expectable

Table 4
Specificity determinants for GEF-CK, CK2 and CK1[a]

	Residue specificity	Crucial position[b]	Specificity determinants at crucial position	Specific peptide substrates
GEF-CK	Ser	$n+2$	Glu > Ser-P	KKIEKFQSEEQQQ
CK2	Ser > Thr	$n+3$	Tyr-P > Glu ≅ Asp ≅ Ser-P > Thr-P ≅ Tyr	RRRADDSDDDDD
CK1	Ser > Thr ≫ Tyr	$n-3$	Ser-P > Thr-P ≫ Asp > Glu	RRKDLHDDEEDEAMSITA

[a]Based on data drawn from this paper as well as from [12,14,19–21].
[b]Relative to that of the phosphorylatable residue ($n \pm 0$).

that casein kinase(s) or related enzymes with the same specificity are also expressed in tissues other than lactating mammary gland, possibly representing a class of Ser-X-Glu/SerP specific secretory pathway protein kinases. This postulate is grounded on the observation that in several secreted phosphoproteins the phosphorylated sites strictly conform to the minimum S-X-E/Sp consensus sequence [2,24,25] which is recognized by GEF-CK but neither by CK2 nor by CK1; it has been also recently corroborated by the detection in rat liver of a CK1/CK2 unrelated protein kinase displaying a specificity similar to that of genuine casein kinase [26].

The main outcomes of the present study, aimed at a better understanding of the casein kinase extracted from the Golgi apparatus of rat lactating mammary gland (GEF-CK), are the following.

1. A specific substrate reproducing the 28–40 sequence of β-casein has been developed for monitoring the activity of casein kinase. This peptide, unlike casein, is not phosphorylated by the other members of the 'casein kinase' family, namely ubiquitous protein kinases CK1 and CK2, while it is phosphorylated more readily than casein itself by GEF-CK (see Table 1). The β[28–40] peptide will prove also useful for the detection and monitoring of 'casein kinases' different from CK1 and CK2 in tissues other than mammary gland [26].

2. The structural requirements of GEF-CK have been further defined by showing that neither aspartic acid nor the phosphoamino acids TyrP and ThrP can substitute for glutamic acid in the consensus sequence S-X-E-X. Only phosphoserine can replace glutamic acid, albeit less efficiently. The failure of aspartic acid to replace glutamic acid as specificity determinant is in agreement with the analysis of the potential sites that are actually phosphorylated in casein fractions [8] and it may account for the observation that potential phosphoacceptor sites in lysozyme, having the S-X-D sequence, could not be phosphorylated by a casein kinase preparation from bovine mammary gland, despite their similarity to sequences readily phosphorylated in α-lactalbumin, displaying however a S-X-E motif [27]. GEF-CK is therefore the member of the 'casein kinase' family endowed with the most stringent specificity, as summarized in Table 4, where the elements contributing to the consensus sequences of CK1, CK2 and GEF-CK are compared. Unlike CK1, which is capable to phosphorylate also tyrosine, under certain circumstances [28], and CK2, which tolerates a variety of acidic and phosphorylated residues as specificity determinants [18], GEF-CK displays the highest selectivity toward serine as opposed to threonine [9] and tolerates only glutamic acid and, to a lesser extent phosphoserine, as specificity determinants.

3. The very low activity of GEF-CK toward the optimal CK2 substrate RRRADDSDDDDD corroborates the concept that this peptide is highly selective for CK2 alone [12]. Consequently three peptide substrates (listed in Table 4) are now available for the specific monitoring of each of the three known members of the 'casein kinases' family, namely GEF-CK, CK1 and CK2.

The new information provided by this study will prove helpful both for the further characterization of GEF-CK and for the detection of 'casein kinases' with similar specificity in organisms and tissues other than the lactating mammary gland.

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References

[1] Kemp, B.E. and Pearson, R.B. (1990) Trends Biochem. Sci. 15, 342–346.
[2] Pinna, L.A., Meggio, F. and Marchiori, F. (1990) in: Peptides and Protein Phosphorylation (Kemp, B.E., Ed.) pp. 145–169, CRC Press, Boca Raton, FL. .
[3] Allende, J.E. and Allende, C.C. (1995) FASEB J. 9, 313–323.
[4] Bingham, E.W. and Groves, M.L. (1979) J. Biol. Chem. 254, 4510–4515.
[5] Szymanski, E.S. and Farrell, Jr., H.M. (1982) Biochim. Biophys. Acta 702, 163–172.
[6] Pascall, J.C., Boulton, A.P. and Craig, R.K. (1981) Eur. J. Biochem. 119, 91–99.
[7] Moore, A., Boulton, A.P., Herd, H.W., Jarasch, E.D. and Craig, R.K. (1985) Eur. J. Biochem. 152, 729–737.
[8] Mercier, J.C. (1981) Biochimie 63, 1–17.
[9] Meggio, F., Boulton, A.P., Marchiori, F., Borin, G., Lennon, D.P.W., Calderan, A. and Pinna, L.A. (1988) Eur. J. Biochem. 177, 281–284.
[10] Meggio, F., Perich, J.W., Meyer, H.E., Hoffmann-Posorske, E., Lennon, D.P.W., Johns, R.B. and Pinna, L.A. (1989) Eur. J. Biochem. 186, 459–464.
[11] Meggio, F., Donella Deana, A. and Pinna, L.A. (1981) J. Biol. Chem. 256, 11958–11961.
[12] Marin, O., Meggio, F. and Pinna, L.A. (1994) Biochem. Biophys. Res. Commun. 198, 898–905.
[13] Sarno, S., Boldyreff, B., Marin, O., Guerra, B., Meggio, F., Issinger, O.G. and Pinna, L.A. (1995) Biochem. Biophys. Res. Commun. 206, 171–179.
[14] Meggio, F., Perich, J.W., Reynolds, E.C. and Pinna, L.A. (1991) FEBS Lett. 279, 307–309.
[15] Glass, D.B., Masaracchia, R.A., Feramisco, J.R. and Kemp, B.E. (1978) Anal. Biochem. 87, 566–575.
[16] Perich, J.W., Meggio, F., Reynolds, E.C., Marin, O. and Pinna, L.A. (1992) Biochemistry 31, 5893–5897.
[17] Ribadeau Dumas, B., Brignon, G., Grosclaude, F. and Mercier, J.-C. (1971) Eur. J. Biochem. 25, 505–514.
[18] Meggio, F., Marin, O. and Pinna, L.A. (1994) Cell. Mol. Biol. Res. 40, 401–409.
[19] Flotow, H., Graves, P.R., Wang, A., Fiol, C.J., Roeske, R.W. and Roach, P.J. (1990) J. Biol. Chem. 265, 14262–14269.
[20] Meggio, F., Perich, J.W., Reynolds, E.C. and Pinna, L.A. (1991) FEBS Lett. 283, 303–306.
[21] Meggio, F., Perich, J.W., Marin, O. and Pinna, L.A. (1992) Biochem. Biophys. Res. Commun. 182, 1460–1465.
[22] Hammarsten, O. (1883) Z. Physiol. Chem. 7, 227.
[23] Hanks, S.K. and Hunter, T. (1995) FASEB J. 9, 576–596.
[24] Sorensen, E.S. and Petersen, T.E. (1994) Biochem. Biophys. Res. Commun. 198, 200–205.
[25] Hu, B., Coulson, L., Moyer, B. and Price, P.A. (1995) J. Biol. Chem. 270, 431–436.
[26] Lasa-Benito, M., Marin, O., Meggio, F. and Pinna, L.A. (1995) 20th European Symposium Hormones and Cell Regulation, Mount Ste., Odile, France, Sept. 1995, Abstr. A-34.
[27] Bingham, E.W., Parris, N. and Farrell, Jr., H.M. (1988) J. Dairy Sci. 71, 324–336.
[28] Hoekstra, M.F., Dhillon, N., Carmel, G., DeMaggio, A.J., Lindberg, R.A., Hunter, T. and Kuret, J. (1994) Mol. Biol. Cell 5, 877–886.