Novel consensus sequence for the Golgi apparatus casein kinase, revealed using proline-rich protein-1 (PRP1)-derived peptide substrates

Anna Maria BRUNATI[2], Oriano MARIN[2], Alfredo BISINELLA, Alessandro SALVIATI and Lorenzo A. PINNA[1]

Dipartimento di Chimica Biologica, Centro per lo Studio delle Biomembrane del CNR and CRIBI, Università degli Studi di Padova, Viale G. Colombo 3, 35121 Padova, Italy

Previous studies have shown that the Golgi apparatus casein kinase (G-CK) recognizes phosphoacceptor sites specified by the triplet SXE/Sp, which is found in several phosphoproteins, besides casein itself. In the present study, we report that G-CK can phosphorylate, with comparable efficiency, sequences surrounding Ser-22 of salivary proline-rich protein-1 (PRP1), which do not conform to the SXE/Sp motif. By using a series of peptide substrates derived from the PRP1 Ser-22 site, we also have shown that the optimal consensus sequence recognized by G-CK in this case was SXQXX(D/E)3, where the acidic residues at positions $n+5$ to $n+7$ and, to a lesser extent, the glutamine residue at position $n+2$ are the critical determinants.

Key words: casein kinase, Golgi apparatus, phosphoproteins, proline-rich protein-1, protein kinases.

INTRODUCTION

The term casein kinase (CK) is often used to indicate several classes of otherwise unrelated Ser/Thr protein kinases that share a preference for casein over histones, protamines and myelin basic protein as a phosphorylatable substrate *in vitro*. This property, unusual among Ser/Thr kinases, reflects the ability of these enzymes to recognize residues specified by acidic and/or phosphorylated, rather than basic or prolyl, side chains nearby. Most CKs, however, are physiologically unrelated to casein, and fall into two sub-families of ubiquitous and pleiotropic enzymes, conventionally termed CK-1 and CK-2, which are implicated in a wide variety of cell functions. CK-1 makes up an independent sub-family of protein kinases [1] with four members in *Saccharomyces cerevisiae*, at least seven in human, and as many as 87 in the nematode *Caenorhabditis elegans* [2]. CK-2 belongs to the CMGC group of protein kinases and is normally present in the cell as a heterotetrameric holoenzyme composed of two catalytic (α- and/or α'-) and two regulatory β-subunits [3,4]. At variance with pleiotropic CK-1 and CK-2, bona fide CK(s) are committed to the phosphorylation of casein fractions before their secretion with milk. Consequently, the best characterized CK has been isolated from the Golgi apparatus of the lactating mammary gland, whence its acronyms, GEF-CK (Golgi-enriched fraction CK) [5] or Golgi apparatus CK (G-CK) derive [6]. Although its primary structure is still unknown, G-CK has been biochemically characterized with special reference to its site specificity, which is similar to but definitely distinct from that of CK-2 [7]. Systematic analysis of phosphorylated sites in a variety of casein fractions [8], in conjunction with studies with synthetic peptide substrates [9–11], led to the definition of the consensus sequence: SXE/Sp-X, where, in contrast to CK-1 and CK-2, threonine cannot effectively replace serine as a phosphoacceptor residue, nor can aspartic acid as effectively replace glutamic acid as a specificity determinant (for details see [11]). Based on this consensus it has been postulated that a number of proteins known to contain the phosphorylated motif(s) SpXE/Sp within sequences that are not recognized by other kinases might be physiological substrates of G-CK-like kinases, although they are expressed in tissues other than the mammary gland (see [12] and references therein). This hypothesis was strongly corroborated by the isolation of an enzyme, biochemically indistinguishable from G-CK, from the Golgi apparatus of rat liver, spleen, brain and kidney [6], and by reports showing that proteins phosphorylated at SXE motifs, e.g. osteopontin [13] and glucose-regulated protein (GRP)-94 [14] are indeed good substrates for G-CK *in vitro*. However the incontrovertible evidence that a protein kinase with the same specificity as G-CK is responsible for the phosphorylation of proteins other than casein in tissues, other than mammary gland, has been provided, to the best of our knowledge, in only one case [15], where it was shown that an acidic proline-rich protein (PRP1) secreted from human salivary gland was phosphorylated at a residue (Ser-8) exhibiting the consensus for both G-CK and CK-2 (SQED), and that the phosphorylation of this residue *in situ* was abrogated by a mutation that destroyed the G-CK consensus but not by a mutation preventing recognition by CK-2. Interestingly, PRP1 is also phosphorylated *in vivo* at another site (Ser-22) [15], where the sequence does not conform to the consensus of G-CK nor to any other known kinase consensus sequence (see Figure 1). This finding could be explained, assuming either the implication of a novel kinase in which the consensus is fulfilled by the sequence around Ser-22 or that G-CK is also able to recognize consensus sequences different from the canonical one, SXE/Sp. In the present work we demonstrate the plausibility of the latter proposition by showing that a peptide reproducing the Ser-22 site of PRP1 is phosphorylated by purified G-CK as effectively as the one reproducing the canonical site (Ser-8), and evidence is provided that the recognition of Ser-22 crucially depends on the glutamine residue at position $n+2$ and, even more stringently, on the acidic triplet, DEE, at positions $n+5$ to $n+7$.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP was purchased from Amersham (Arlington Heights, U.S.A.). All other chemicals were from Sigma (Poole, Dorset,

Abbreviations used: CK, casein kinase; G-CK, Golgi apparatus CK; PRP1, proline-rich protein-1; GRP, glucose-regulated protein.

[1] To whom correspondence should be addressed (e-mail pinna@civ.bio.unipd.it).

[2] These authors contributed equally to this work.

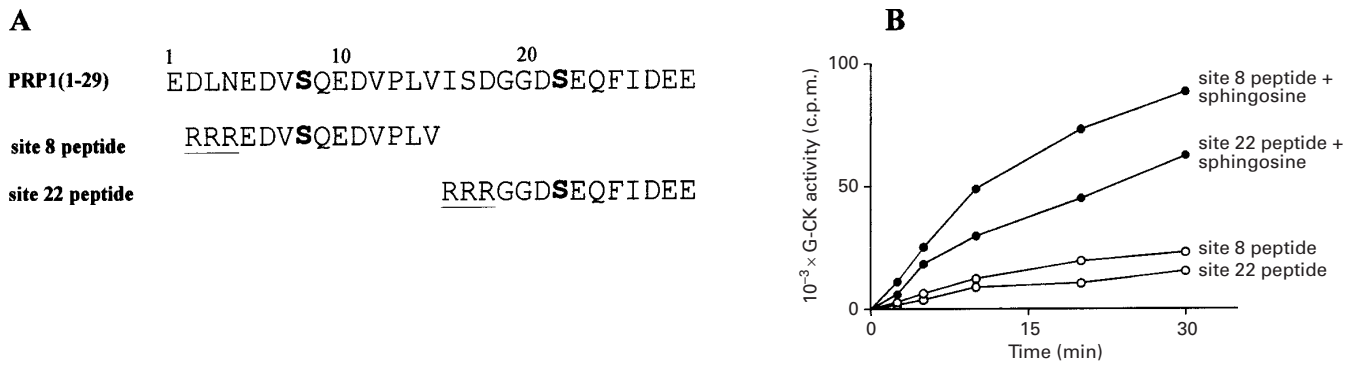

Figure 1 Phosphorylation of peptides derived from sites 8 and 22 of PRP1 by G-CK

(**A**) The N-terminal segment (1–29) of PRP1 is shown with the two phosphoacceptor sites, Ser-8 and Ser-22, in bold. Three arginine residues (underlined) were attached at the N-terminus of the site-8 and the site-22 peptides to allow the evaluation of radiophospho-labelling using the phosphocellulose paper procedure [17]. These arginine residues did not significantly influence phosphorylation by G-CK (see below and Table 1). (**B**) Time course of phosphorylation of site-8 and site-22 peptides (each at 750 μM) by G-CK in the absence (○) or presence of 50 μM sphingosine (●).

U.K.). Synthetic peptides, corresponding to the phosphorylation sites Ser-22 and Ser-8 of the PRP-1 protein and their analogues, were prepared on a peptide synthesizer (431 A; Applied Biosystems) using fluoren-9-methoxycarbonyl (Fmoc) chemistry with an *N*-methylpyrrolidone solvent system [16]. Chain assembly was performed on 0.1 mmol of Wang resin (0.96 mmol/g) (Novabiochem) using Fmoc-protected amino acids activated with a mixture of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, *N*-hydroxybenzotriazole and *N*-ethyldi-isopropylamine in dimethylformamide. Side-chain protecting groups were removed during cleavage with a solution containing 95 % (v/v) trifluoroacetic acid, 3 % (v/v) anisole, 1 % (v/v) 1,2-ethanedithiol and 1 % (v/v) ethyl methyl sulphide. After cleavage, peptides were purified to homogeneity by reverse-phase HPLC using a linear gradient of 0–40 % acetonitrile. Purity of the synthetic peptides was assessed using matrix-assisted laser-desorption ionization ('MALDI') MS. The other peptides were synthesized as described previously [11,14]

Purification of G-CK

G-CK was isolated from the Golgi fraction of rat lactating mammary gland [6] and purified so that it was free of other contaminating kinase activities, as judged by in-gel phosphorylation experiments described previously [14]. After removal of contaminating GRP-94 protein by concanavalin A treatment [14], the enzyme was > 80 % pure, as judged from the intensity of the 170-kDa band revealed by Coomassie Brilliant Blue staining.

Phosphorylation assays

G-CK activity was routinally assayed using the specific peptide substrate β(28–40) as described previously [11]. Phosphorylation of peptides and kinetic analyses were performed as in [6], except for the omission of $MgCl_2$ from the incubation medium. Phosphorylation was evaluated by the phosphocellulose paper method, whenever applicable, and, in the case of peptides with less than three basic residues, a combination of the Ag1-X8 chromatography and acid hydrolysis methods was employed (detailed in protocols 2 and 4 respectively of [17]).

RESULTS AND DISCUSSION

Figure 1(A) shows the sequence of the N-terminal segment of PRP1, including the two phosphorylated residues, Ser-8 and Ser-22. Although the former fulfils the consensus sequence of G-CK (SXE) by virtue of Glu-10, the latter does not, having a glutamine instead of a glutamic acid residue at the crucial $n+2$ position (Gln-24). *In vivo* studies have confirmed that Glu-10 is essential for the phosphorylation of Ser-8, whereas the phosphorylation of Ser-22 was suppressed by the replacement of the three acidic residues, Asp-27, Glu-28 and Glu-29 with alanine residues [15]. To check whether and eventually to what extent both sites are susceptible to phosphorylation by G-CK, two peptides encompassing the sequences surrounding Ser-8 and Ser-22 from position $n-3$ to position $n+7$ were synthesized (Figure 1A, site-8 peptide and site-22 peptide), with an additional triplet of arginine residues at the N-terminus, so that the phosphorylation assay based on phosphocellulose paper could be used. The time course of phosphorylation of the two peptides at 750 μM by highly purified G-CK from lactating mammary gland (Figure 1B) shows that both were good substrates, the phosphorylation of the site-8 peptide proceeding slightly faster than that of the site-22 peptide. By prolonged incubation of the peptides (50 μM) with 500 μM (instead of 50 μM) ATP, up to 0.50 mol and 0.43 mol of phosphate/mol was incorporated into the site-8 and site-22 peptides respectively. Under similar conditions, the specific β-casein peptide, routinely used for monitoring G-CK activity, incorporated 0.47 mol of phosphate/mol of peptide. These data are consistent with the observation that, although PRP1 is fully phosphorylated at both sites *in vivo* [18], if PRP1 is transfected into a human submandibular cell line phosphorylation of Ser-22 is less exhaustive than phosphorylation of Ser-8 [15]. Figure 1(B) also shows that the phosphorylation of both PRP1 peptides is equally stimulated by sphingosine, which recently has been found to act as a potent activator of G-CK when tested on a variety of protein and peptide substrates (A. M. Brunati and L. A. Pinna, unpublished work). This observation, in conjunction with the finding that the PRP1 site-22 peptide competes with G-CK for the phosphorylation of casein (results not shown), corroborates the concept that the same kinase is implicated in the phosphorylation of both PRP1 peptides. It can be concluded therefore that the site-22 sequence, although it does not conform to the canonical consensus of G-CK, is nevertheless phosphorylated by G-CK, which makes plausible the involvement of a G-CK-like kinase in the phosphorylation of both sites of PRP1 *in vivo*.

In order to understand which are the regional structural features that make Ser-22 susceptible to G-CK, despite its lack of

Table 1 Kinetic constants for the phosphorylation of peptide substrates by G-CK

The phosphoacceptor residue is shown in bold type. Underlining denotes substitutions relative to the sequence in the parent protein. Values are the means of at least three independent determinations with an S.E.M. < 0.15. AQP2, aquaporin-2; n.d., not determined because the phosphorylation rate was below the level of detection. The results shown were derived from the present study; similar data for the phosphorylation of peptides 12, 13 and 17 by G-CK has been reported previously [11,14].

Peptide	Sequence	Parent protein	V_{max} (nmol · min^{-1} · mg^{-1} of protein)	K_m (mM)	V_{max}/K_m
1	RRRGGD**S**EQFIDEE	PRP1 (site-22)	4.78	0.30	15.93
2	RRRGGD**S**AQFIDEE		6.90	0.33	20.90
3	RRRGGA**S**EQFIDEE		4.21	0.40	10.52
4	RRRGGD**S**EEFIDEE		7.13	0.66	10.80
5	RRRGGD**S**EAFIDEE		0.91	0.87	1.12
6	RRRGGD**S**EQFIAAA		< 0.5	n.d.	n.d.
7	RRRGGD**S**EQFIAEE		1.47	0.40	3.67
8	RRRGGD**S**EQGGDEE		4.51	0.31	14.54
9	EEEGGD**S**EQFIDEE		4.88	0.35	13.94
10	RRREDV**S**QEDVPLV	PRP1 (site-8)	7.15	0.70	10.21
11	RRREDV**S**QQDVPLV		< 0.5	n.d.	n.d.
12	KWQRP**S**KEVEEDE	GRP-94 (321–333)	2.96	0.15	19.73
13	KYKAF**S**KEADDPM	GRP-94 (337–349)	14.47	1.24	11.66
14	VRRRQ**S**VELH	AQP2 (251–269)	2.33	0.18	12.94
15	VRRRQ**S**KELH		3.71	0.23	16.10
16	VRRRQ**S**VALH		< 0.5	n.d.	n.d.
17	KKIEKFQ**S**EEQQQ	β-casein (28–40)	6.34	0.41	15.46
18	KKIEKFQ**S**EAQQQ		< 0.5	n.d.	n.d.

the canonical consensus, the site-22 peptide was modified by changing a number of potentially important amino acids and the efficiency of resulting derivatives as G-CK substrates was compared with that of the parent peptide. The kinetic constants are shown in Table 1, together with those of a number of peptide substrates displaying the canonical consensus for G-CK (SXE). It can be seen that the phosphorylation efficiency (V_{max}/K_m) of the site-22 peptide (peptide 1) was even higher than that of the site-8 peptide (peptide 10), by virtue of a significantly lower K_m, although its phosphorylation rate was also lower at saturation (V_{max} 4.78 compared with 7.15 nmol · min^{-1} · mg^{-1} protein). It is also noteworthy that the phosphorylation efficiency of the site-22 peptide was similar to that of the β-casein 28–40 peptide, which is routinally used for monitoring G-CK activity [6]. This activity was not due to the glutamic acid residue at position $n+1$ (which could have been suspected to be a surrogate for the crucial residue lacking at position $n+2$), since its replacement with alanine did not decrease but, rather, slightly increased phosphorylation efficiency (Table 1, compare peptides 1 and 2). This is in agreement with the observation that a basic rather than acidic residue is found at position $n+1$ in all of the putative G-CK phosphorylation sites of GRP-94 [14] (e.g. peptides 12 and 13 in Table 1), and that if the valine at position $n+1$ of the putative phosphorylation site of aquaporin-2 (Ser-256) was replaced by lysine the resulting peptide would be phosphorylated faster than the parent peptide (Table 1, compare peptides 14 and 15). Collectively, these data support the view that the nature of the residue at position $n+1$ is not important, either at canonical sites or at the atypical PRP1 site 22. Likewise, the aspartate residue adjacent to the N-terminal side of Ser-22 is also dispensable, as it can be replaced by alanine without a dramatic decrease in phosphorylation efficiency (Table 1, compare peptides 1 and 3).

Somewhat surprisingly, the replacement of glutamine for glutamic acid at the crucial $n+2$ position, which restores the canonical consensus in the site-22 peptide, did not improve the phosphorylation efficiency (Table 1, compare peptides 1 and 4), which is consistent with the view that the phosphorylation of this site is already optimized by other features. The replacement of this glutamine residue with alanine, however, was detrimental, causing a five-fold decrease in V_{max} and a more than two-fold increase in K_m (Table 1, compare peptides 1 and 5). Much more dramatic was the consequence of replacing the acidic triplet, DEE (Asp-Glu-Glu), at positions $n+5$ to $n+7$ with alanine residues; this peptide (number 6 in Table 1) was unable to be phosphorylated by G-CK, but replacement of the first residue of the triplet (Asp-27) alone resulted in detectable, albeit drastically decreased, phosphorylation (Table 1, peptide 7). It seems likely, therefore, that the whole acidic triplet together with the glutamine residue at position $n+2$ are required for optimal phosphorylation of the Ser-22 site, since the replacement of one of these residues is sufficient to drastically decrease, but not abolish completely, phosphorylation. In contrast, the phosphorylation of canonical sites crucially relies on the individual glutamate residue at position $n+2$ (Table 1), seen by the dramatic effect of its substitution with alanine in β-casein and in the aquaporin-2-derived peptide (compare peptides 18 and 16 with peptides 17 and 14 respectively). Even the more conservative substitution of glutamic acid with glutamine within the canonical consensus, SXE, was not tolerated, as shown with the PRP-1 site-8 peptide (peptide 10), where this individual substitution, giving rise to peptide 11, prevented any detectable phosphorylation. This observation provides clear-cut evidence that the phosphorylation of the PRP-1 Ser-22 site by G-CK is based on structural determinants which are different from those specific for the phosphorylation by G-CK of the Ser-8 site and, in general, of the canonical sites. Apparently, the nature of the two bulky non-polar residues found at positions $n+3$ and $n+4$ in the Ser-22 site (Phe-Ile) is not so important for phosphorylation, since peptide 8, in which each of these residues was replaced by glycine, was as good a substrate as the parent peptide (see Table 1).

Finally, it must be emphasized that the three arginine residues introduced at the N-terminal end of the peptide substrates to make the phosphorylation assay with phosphocellulose paper possible have no influence on the phosphorylation reaction, since a derivative of the PRP-1 site-22 peptide (peptide 1), in which these three arginines were replaced by glutamic acid residues (peptide 9), was phosphorylated with comparable efficiency. It should also be noted in this connection that multiple basic

Table 2 Potential phosphoacceptor sites resembling the PRP1 Ser-22 site

Peptides characterized by the motif **SXQ** and by two or more acidic residues (also shown in bold) at positions spanning $n+4$ to $n+7$, relative to a serine residue (bold and underlined) were identified using a SwissProt database search, and a subset of proteins containing the motif were selected.

Sequence	Protein	Position of serine residue
GD**S**E**Q**FI**DEE**	PRP1 (*Homo sapiens*)	22
SE**S**E**Q**S**EED**G	β-NAP protein (*H. sapiens*)	734
SE**S**E**Q**S**EED**G	AP-3 protein (*H. sapiens*)	734
AA**S**E**Q**YI**DTE**	Intersectin-2 long isoform (*H. sapiens*)	1542
ME**S**E**Q**SA**DE**V	CD39 antigen (*Mus musculus*)	142
GA**S**E**Q**YI**DTE**	Ese 2L (*M. musculus*)	1504
LISE**Q**FI**D**Q**E**	Polypeptide deformilase (*Urea urelyticum*)	149
GD**S**A**Q**FVS**DE**	Pentafuntional arom polypeptide (*Saccharomyces cerevisiae*)	386
GN**S**E**Q**FV**D**N**D**	CG100563 gene product (*Drosophila melanogaster*)	463
ED**S**E**Q**Q**EEE**Q	CG15073 gene product (*D. melanogaster*)	96
GI**S**E**Q**LI**D**S**D**	Annezin max3 (*Oryzias latipes*)	185
GE**S**K**Q**FA**EE**I	Heat shock 70-kD (*Dauca carota*)	113
YY**S**E**Q**YE**D**N**D**	NisT protein (*Lactococcus lactis*)	592
ES**S**D**Q**ED**DDD**	T29A15.130 hypothetical protein (*Arabidopsis thaliana*)	305
GE**S**E**Q**IA**DED**	AC009606 hypothetical protein (*A. thaliana*)	48
LD**S**E**Q**E**DED**A	B0511.12 (*Caenorhabdatis elegans*)	1082

residues are present upstream from the target serine in several phosphoacceptor sites specified by the canonical consensus, e.g. in β-casein (peptide 17), aquaporin-2 (peptide 14) and GRP-94 (peptides 12 and 13). In addition, it is noteworthy that an arginine at positions $n-2$ to $n-4$ relative to a phosphorylated serine specifies the sequence context which is recognized by the 14-3-3 proteins [19]. Thus it is possible that, in some circumstances, phosphorylation by G-CK may trigger the association of phosphoacceptors proteins with 14-3-3 proteins.

Conclusions

The main conclusion to be drawn from the present study is that the classical consensus sequence SXE/Sp used to predict the phosphoacceptor sites for G-CK is not the only one recognized by this kinase, since a peptide where the sequences around PRP1 Ser-22 were reproduced was phosphorylated equally well, although it lacked the SXE/Sp motif. As a consequence of this finding, it appears that Ser-22 is phosphorylated by the same G-CK-like kinase shown to be responsible for the phosphorylation of Ser-8 within full-length PRP1 [15]. *In vivo*, the phosphorylation of Ser-22 was abolished by the replacement of the acidic triplet at positions $n+5$ to $n+7$ with three identical amino acid residues [15], which, when performed in the present study, suppressed phosphorylation of the derived peptide by G-CK (see Table 1). The present data would therefore indicate that the novel consensus sequence, which is phosphorylated by G-CK with an efficiency similar to the canonical one, is SXQXXDEE. Apparently, a combination of the two motifs does not potentiate phosphorylation, as judged from the lack of effect when Gln ($n+2$) was replaced by Glu in the site-22 peptide. By contrast, the replacement of glutamic acid by glutamine at position $n+2$ in the site-8 peptide prevented phosphorylation, showing that the negative charge of glutamic acid at position $n+2$ is essential for recognition of the canonical sequence, whereas it is dispensable in the PRP1 site-22 consensus.

A search of the SwissProt database revealed a number of proteins with potential phosphoacceptor sites resembling the new consensus outlined here. Some of these, characterized by the motif SXQ and by two or more acidic residues at positions spanning $n+4$ to $n+7$, are listed in Table 2. Interestingly, in at least two cases, human β-NAP [20] and human AP-3 [21], the proteins have been reported to undergo phosphorylation.

The authors are grateful to Dr Anders Bennick (University of Toronto, Canada) for making available unpublished data. This work was supported by the Armenise-Harvard Foundation, A. I. R. C., the Italian Ministry of Health (Project AIDS), the Italian M. U. R. S. T. (PRIN 1997) and C. N. R. (Target Project on biotechnology)

REFERENCES

1 Gross, S. D. and Anderson, R. A. (1998) Casein kinase 1: spatial organization and positioning of a multifunctional protein kinase family. Cell. Signalling **10**, 699–711
2 Plowman, G. D., Sudarsanam, S., Bingham, J., Whyte, D. and Hunter, T. (1999) The protein kinases of *C. elegans*: a model for signal transduction in multicellular organisms. Proc. Natl. Acad. Sci. U.S.A. **96**, 13603–13610
3 Pinna, L. A. and Meggio, F. (1997) Protein kinase CK2 (casein kinase 2) and its implication in cell division and proliferation. Prog. Cell Cycle Res. **3**, 77–97
4 Guerra, B. and Issinger, O. G. (1999) Protein kinase CK2 and its role in cellular proliferation, development and pathology. Electrophoresis **20**, 391–408
5 Moore, A., Boulton, A. P., Herd, H. W., Jarash, E. D. and Craig, R. K. (1995) Purification and tissue-specific expression of casein kinase from the lactating guinea pig mammary gland. Eur. J. Biochem. **152**, 729–737
6 Lasa, M., Marin, O. and Pinna, L. A. (1997) Rat liver Golgi apparatus contains a protein kinase similar to the casein kinase of lactating mammary gland. Eur. J. Biochem. **243**, 719–725
7 Pinna, L. A. and Ruzzene, M. (1996) How do protein kinases recognize their substrates? Biochim. Biophys. Acta **1314**, 191–225
8 Mercier, J. C. (1981) Phosphorylation of casein, present evidence for an amino acid triplet code posttranslationally recognized by specific kinase. Biochimie **63**, 1–17
9 Meggio, F., Boulton, A. P., Marchiori, F., Borin, G., Lennon, D. W., Calderan, A. and Pinna, L. A. (1989) Substrate-specificity determinants for a membrane-bound casein kinase of lactating mammary gland. A study with synthetic peptides. Eur. J. Biochem. **177**, 281–284
10 Meggio, F., Perich, J. W., Meyer, H. E., Hoffmann-Posorske, E., Lennon, D. P. W., Johns, B. R. and Pinna, L. A. (1988) Synthetic fragments of β-casein as model substrates for liver and mammary gland casein kinases. Eur. J. Biochem. **186**, 459–464
11 Lasa-Benito, M., Marin, O., Meggio, F. and Pinna, L. A. (1996) Golgi apparatus mammary gland casein kinase: monitoring by a specific peptide substrate and definition of specificity determinants. FEBS Lett. **382**, 149–152
12 Price, P. A., Rice, J. S. and Williamson, M. K. (1994) Conserved phosphorylation of serine in the Ser-X-Glu/Ser(P) sequence of the vitamin K-dependent matrix Gla protein from shark, lamb, rat, cow and human. Protein Sci. **3**, 822–830
13 Lasa, M., Chang, P. L., Prince, C. W. and Pinna, L. A. (1997) Phosphorylation of osteopontin by Golgi apparatus casein kinase. Biochem. Biophys. Res. Commun. **240**, 602–605
14 Brunati, A. M., Contri, A., Muenchbach, M., James, P., Marin, O. and Pinna, L. A. (2000) GRP94 (endoplasmin) co-purifies with and is phosphorylated by Golgi apparatus casein kinase. FEBS Lett. **471**, 151–155
15 Drzymala, L., Castle, A., Cheung, J. C. and Bennick, A. (2000) Cellular phosphorylation of an acid proline-rich protein, PRP1, a secreted salivary phosphoprotein. Biochemistry **39**, 2023–2030
16 Fields, G. B. and Noble, R. L. (1990) Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonilamino acid. Int. J. Pept. Protein Res. **35**, 161–214
17 Ruzzene, M. and Pinna, L. A. (1999) Assay of protein kinases and phosphatases using specific peptide substrates. In Protein Phosphorylation: A Practical Approach, 2nd edn, (Hardie, D. G., ed.), pp. 221–253, Oxford University Press, Oxford
18 Wong, R. S. C. and Bennick, A. (1980) The primary structure of a salivary calcium-binding proline-rich phosphoprotein (protein C), a possible precursor of a related salivary protein A. J. Biol. Chem. **255**, 5943–5948
19 Aitken, A. (1996) 14-3-3 and its possible role in co-ordinating multiple signalling pathways. Trends Cell Biol. **6**, 341–347
20 Newman, L. S., McKeever, M. O., Okano, H. J. and Darnell, R. B. (1995) β-NAP, a cerebellar degeneration antigen, is a neuron-specific vesicle coat protein. Cell **82**, 773–783
21 Murphy, J. E., Hanover, J. A., Froehlich, M., DuBois, G. and Keen, J. H. (1994) Clathrin assembly protein AP-3 is phosphorylated and glycosylated on the 50-kDa structural domain. J. Biol. Chem. **269**, 21346–21352

Received 12 June 2000/27 July 2000; accepted 18 August 2000