

## Purification of Golgi casein kinase from bovine milk

Jennifer S. DUNCAN<sup>\*1</sup>, Mark C. WILKINSON<sup>‡</sup> and Robert D. BURGOYNE<sup>\*2</sup>

<sup>\*</sup>The Physiological Laboratory, University of Liverpool, Crown Street, Liverpool L69 3BX, U.K., and <sup>‡</sup>School of Biological Sciences, University of Liverpool, Crown Street, Liverpool L69 3BX, U.K.

Caseins and many other secretory proteins are phosphorylated during their transport through the secretory pathway by a protein kinase present within Golgi compartments. Molecular analysis of the Golgi casein kinase (GCK) has not been possible since it has not been purified to homogeneity or been cloned. Previous attempts have been made to purify GCK activity from mammary gland Golgi fractions, but these have not resulted in extensive purification of the enzyme. In the present study, we have demonstrated that substantial amounts of GCK activity, assayed using a specific peptide substrate, can be detected as a soluble form in bovine milk, and we have used milk as a source

for purification. A purification protocol was established that allowed > 80 000-fold purification to a specific activity of GCK (approx. 700 nmoles/min per mg of protein) far higher than previously achieved. These findings cast doubts on previous claims for purification of GCK activity. In addition, ion-exchange chromatography resolved two closely eluting peaks of activity, suggesting the existence of two related, but distinct, GCK activities.

**Key words:** milk protein, phosphorylation, secretion, secretory pathway.

### INTRODUCTION

The caseins are the predominant milk proteins [1], and it has long been known that they are phosphorylated in lactating mammary epithelial cells shortly after their synthesis and export from the endoplasmic reticulum [2]. The phosphorylation of caseins is important to allow them to bind Ca<sup>2+</sup> and for the subsequent formation of casein micelles, which remain stable in milk [3]. The protein kinase responsible for this physiological casein phosphorylation is present within Golgi compartments [4–8], and is only accessible in *in vitro* assays after disruption of the Golgi membranes [6,9–11]. The use of caseins as substrates for protein kinases has resulted in the identification and detailed characterization of two protein kinases: casein kinase (CK) 1 and CK2 [12]. These are cytosolic enzymes and are, therefore, misnamed (since they would not encounter casein as a physiological substrate). The basic properties of the Golgi casein kinase (GCK) have been characterized in Golgi fractions and membrane extracts [6,9–11], but this enzyme has not been convincingly purified or cloned. In addition, it is neither clear whether GCK activity encompasses single or multiple enzymes, nor is it known if a mammary-gland-specific GCK exists, or if instead GCK is a single, ubiquitously expressed enzyme.

Further analysis of GCKs is of general importance as enzymes with similar activities have been identified in Golgi fractions of various tissues, including liver, spleen, kidney and brain [13]. In addition, a large number of unrelated secretory proteins with various functions have been shown to be phosphorylated during transport through the secretory pathway, including chromogranin B and secretogranin II [14], the enkephalin precursor [15], progastrin [16], osteopontin [17], sialyltransferase [18], and biologically active hormones such as prolactin [19], insulin-like growth factor-binding protein-1 [20] and many others. The significance of the phosphorylation of these proteins is generally

unknown, but is believed to regulate the subsequent proteolytic processing of certain hormone precursors such as progastrin [21]. Phosphorylation of casein by GCK occurs on serine residues within Ser-Xaa-Glu or Ser-Xaa-SerP motifs, or on threonine residues within similar motifs [22]. Phosphorylation of other secretory proteins occurs at the same motifs (e.g. [16]), suggesting that a common GCK could phosphorylate many secretory proteins. In support of this interpretation, crude GCK prepared from mammary gland can efficiently phosphorylate progastrin [23] and osteopontin [17], and progastrin can be phosphorylated by Golgi extracts from various other cell types, including chromaffin and GH3 cells [24]. Determination of whether or not a single widely expressed enzyme phosphorylates all secreted substrates will require identification of the kinase(s) responsible.

GCK has not yet been characterized at a molecular level. An attempt to purify GCK from mammary Golgi fractions by ATP-affinity chromatography was reported, and a 70 kDa protein was claimed to represent the enzyme [25,26]. Surprisingly, immunocytochemical studies suggested that the 70 kDa protein is expressed only in mammary epithelial cells [25], a finding inconsistent with the possible general expression of GCK. This protein was, however, poorly characterized and its identity as GCK was not confirmed. Assays for GCK can be contaminated by the presence of cytosolic CK1 and CK2. An important subsequent advance, therefore, was the development of a synthetic peptide substrate for GCK based on a phosphorylation site in bovine  $\beta$ -casein, and the demonstration that this peptide is an extremely poor substrate for cytosolic CK1 and CK2 [27]. The peptide substrate was recently used to reveal the presence of a GCK activity in liver Golgi fractions, and to follow its partial purification from liver and mammary gland [13]. This resulted in the claim that a 170 kDa polypeptide present in active fractions represented GCK, but it was not demonstrated that this polypeptide co-chromatographed with GCK activity, or that it

Abbreviations used: CK casein kinase; GCK, Golgi casein kinase.

<sup>1</sup> Present address: Easter Bush Veterinary Centre, Royal Dick School of Veterinary Studies, Department of Veterinary Clinical Studies, University of Edinburgh, Roslin EH25 9RG, Midlothian, Scotland. U.K.

<sup>2</sup> To whom correspondence should be addressed (e-mail [burgoyne@liverpool.ac.uk](mailto:burgoyne@liverpool.ac.uk)).

represented a fully purified GCK enzyme. Previous characterization and attempted purification of GCK has been based on the assumption that it is an integral membrane protein of the Golgi, and has used detergent extracts of Golgi fractions as the source of GCK; the possibility of the existence of a secreted soluble form of GCK has not been considered. In the present paper we report the presence of a specific soluble GCK activity in bovine milk, and using this source describe a protocol for substantial purification of GCK to specific activities considerably greater than previously reported. Ion-exchange chromatography, used during this purification, suggested the existence of two separable GCK activities in bovine milk.

## MATERIALS AND METHODS

### Assay of GCK activity

The assay for GCK was modified from a previously described method [28]. Duplicate 5  $\mu$ l aliquots of the test sample were incubated in buffer containing 80 mM Mops (pH 6.3), 15 mM MgCl<sub>2</sub>, 15 mM MnCl<sub>2</sub>, 100  $\mu$ M ATP, 1  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (specific radioactivity 30 Ci  $\cdot$  mmol<sup>-1</sup>). A 1 mM aliquot of the peptide KKIEKFSQEEQQQ was added to half the samples, and the final volume in each assay was 50  $\mu$ l. The reaction was started by the addition of the test sample. The assay mixture was incubated for one hour at 35 °C, the reaction was terminated by the addition of 50  $\mu$ l of 60% (v/v) acetic acid, and duplicate 40  $\mu$ l aliquots of the mixture were then blotted on to individual squares of P81 phosphocellulose paper (Whatman). The papers were washed in 30% acetic acid for 15 min, followed by a further three washes in 15% acetic acid, with a final wash for 5 min in acetone. After drying, the papers were placed in scintillation vials with 10 ml of Cocktail T (BDH), and bound radioactivity was measured in a scintillation counter. For each sample a value was obtained for incorporation of <sup>32</sup>P into the peptide substrate plus background phosphorylation, and for background phosphorylation alone. For each assay a value for peptide autophosphorylation was also obtained. Specific GCK activity was determined after subtraction of background and autophosphorylation values.

### Purification of GCK activity

All procedures were carried out at 4 °C, and all chromatography was performed using a Pharmacia FPLC system. Column fractions were assayed for GCK activity and protein concentration, and analysed by SDS/PAGE. Under the conditions used, all GCK assays were within the linear range of the assay.

Fresh bovine milk was collected directly from the cow, or from the chilled bulk tank, and processed on the same day. Cellular debris and fat were removed from the milk by centrifugation at 800 *g* for 20 min in a Centrikon T-42K bench centrifuge (Kontron Instruments, Watford, Herts., U.K.). The supernatant was collected and centrifuged in a Centrikon T-1170 centrifuge (Kontron Instruments) at 100 000 *g* for 1 h, to leave a casein pellet and a whey supernatant. This supernatant was centrifuged for a further hour at 100 000 *g*, before dialysis of the supernatant against the loading buffer for the first column. This buffer contained 20 mM Mops (pH 7.0), 3 mM MgCl<sub>2</sub> and 1.5 mM EGTA (buffer A), to which 1 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added for phenyl-Sepharose chromatography. The whey was dialysed typically for 16 h, the dialysate centrifuged for 1 h at 100 000 *g*, and the supernatant filtered through 5  $\mu$ m, 1.2  $\mu$ m and 0.45  $\mu$ m syringe filters (Gelman Sciences, Ann Arbor, MI, U.S.A.) before application to the first column.

For tests of ATP-agarose chromatography for the purification of GCK activity, whey was initially fractionated by ion-exchange chromatography on Q-Sepharose. The whey was applied to a 22 ml HiLoad 16/10 Q-Sepharose HP column (Pharmacia) equilibrated in buffer A. Bound proteins were eluted with a 120 ml 0–1.0 M NaCl gradient in buffer A, and 3 ml fractions were collected at a flow rate of 1 ml/min. Active fractions eluting at approx. 300–400 mM NaCl were collected, dialysed against buffer A plus 50 mM NaCl, and applied to a column of  $\gamma$ -phosphate-linked ATP-agarose (Sigma). Bound proteins were eluted with 10 mM MgATP, followed by 1 M NaCl, and 1 ml fractions were collected.

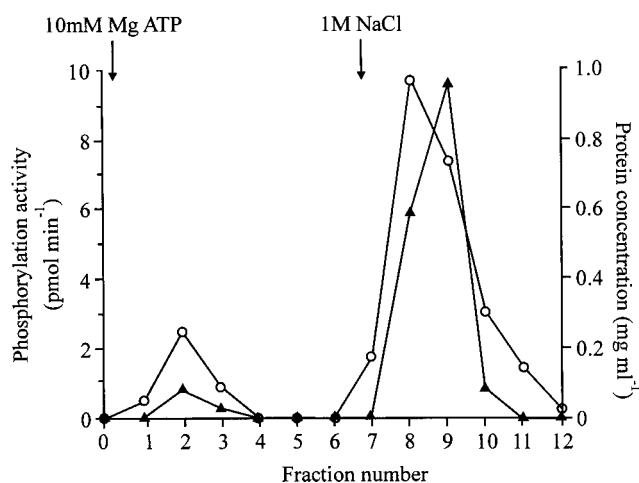
The optimized purification protocol involved sequential chromatography of the whey fraction on phenyl-Sepharose, heparin-Sepharose and Mono Q columns. The phenyl-Sepharose column used was a 22 ml HiLoad 16/10 phenyl-Sepharose HP column (Pharmacia). The column was equilibrated in buffer A plus 1 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Bound proteins were eluted with a 48 ml decreasing 1.0–0 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> gradient. Samples were collected in 3 ml fractions at a flow rate of 1 ml/min. The heparin column was a 5 ml HiTrap heparin-Sepharose column (Pharmacia), which was equilibrated in buffer A with 10% glycerol and 50 mM NaCl. Fractions from the phenyl-Sepharose column were made up to 50 mM NaCl by slow addition of NaCl and gentle mixing. The sample was then applied to the column and bound proteins were eluted in a 90 ml increasing gradient of 50 mM–1 M NaCl in buffer A (also containing 10% glycerol), at a flow rate 1 ml/min. Fractions were collected in 3 ml volumes and dialysed for 3 h against buffer A plus 10% glycerol, and applied to a Mono Q HR 5/5 column (Pharmacia) equilibrated in buffer A containing 10% glycerol. Elution of bound proteins was achieved using an increasing 0–1 M NaCl gradient, with 0.5 ml fractions collected at a flow rate of 0.5 ml/min.

### Peptide sequencing

Using a method adapted from Rosenfeld et al. [29], gel slices from SDS/PAGE were washed (2  $\times$  30 min) with 50% acetonitrile/0.2 M ammonium bicarbonate (pH 8.9), and then freeze-dried. The slices were re-swollen in 0.2 M ammonium bicarbonate (pH 7.8)/0.02% Tween 20 containing trypsin (substrate/trypsin ratio of 30:1), and incubated at 37 °C overnight. Excess buffer was then removed to a second Eppendorf tube, and peptides were extracted from the gel slices with two lots of 60% acetonitrile/0.1% trifluoroacetic acid. The latter and the excess buffer were pooled, concentrated, and applied to a reverse-phase HPLC column to separate the peptides. Suitable peptides were then subjected to N-terminal sequencing by Edman degradation using an Applied Biosystems model 471A Protein Sequenator.

## RESULTS

Previous work has shown that GCK activity present within Golgi fractions is exposed to the lumen and can be only assayed with exogenous substrate following detergent solubilization of the membranes [6,9–11]. This resulted in the assumption that GCK is an integral Golgi membrane protein [25,26]. In our initial experiments, GCK activity was solubilized from lactating mammary tissue using 1% Triton X-100 as previously described [6,9–11]. Extensive dialysis to remove detergent led to massive precipitation of solubilized protein. It was found, however, that GCK activity remained essentially fully soluble. This suggested that GCK may be a soluble luminal enzyme rather than a Golgi transmembrane protein. Theoretically, a Golgi luminal enzyme could be secreted, to at least some extent, into milk along with secretory proteins. We therefore examined the possible presence

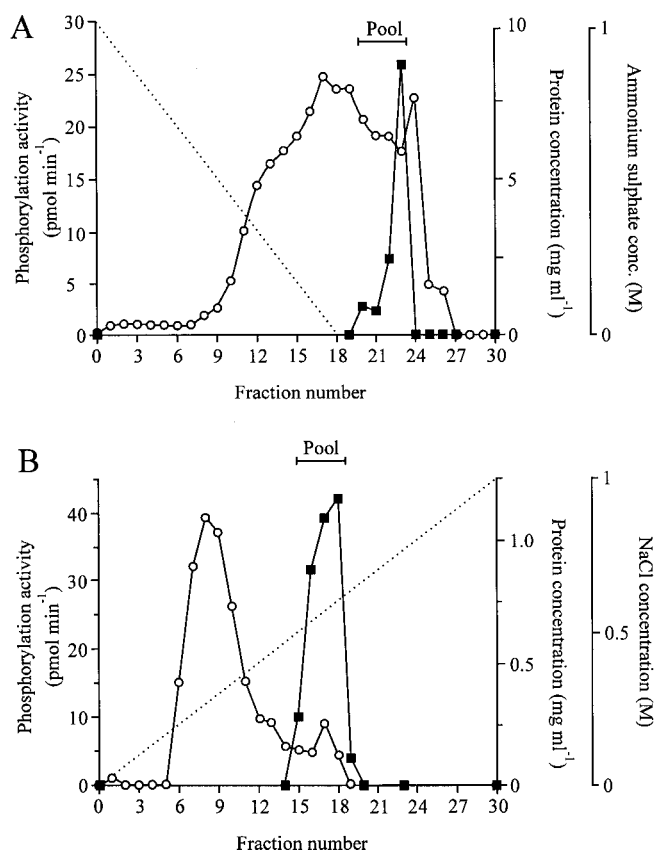


**Figure 1** Fractionation of GCK activity using ATP-agarose

Dialysed whey was fractionated by Q-Sepharose chromatography, and pooled active fractions loaded on to an ATP-agarose column. Bound proteins were eluted with 10 mM MgATP, followed by 1 M NaCl as indicated, and fractions were collected and assayed for protein concentration (○) and GCK activity (▲).

of GCK activity in unpasteurized bovine milk. In these experiments authentic GCK was assayed using a specific  $\beta$ -casein peptide substrate. The milk was centrifuged at low speed to remove any cellular debris prior to assay, and at 100 000 *g* for 1 h to sediment casein micelles and leave the whey supernatant. GCK activity was readily detected in milk and whey fractions with approx. 5-fold enrichment of activity in whey. GCK in milk had a specific activity of up to 30.6 pmol/min per mg of protein. This was lower than the maximum specific activity detected in mammary Golgi extracts using the same substrate peptide ([27], and results not shown), but higher than the specific activity reported in purified liver Golgi fractions, which have also been used as a source of GCK for purification [13]. We decided, therefore, to use bovine milk as the starting material for purification of GCK, as this would provide an easily obtainable source available in large quantities.

It has been claimed that GCK can be purified from mammary Golgi extracts in a single step using affinity chromatography on ATP resins [25,26]. Attempts were made to use ATP-affinity chromatography to purify GCK from whey, based on the previous use of this method. Partial fractionation of whey, by ion-exchange chromatography on Q-Sepharose was initially carried out, and active fractions applied to ATP-agarose columns. Four types of ATP-agarose resin were tested, which differed in their mode of ATP linkage, but GCK activity was found to bind to only one type of resin. GCK activity was bound and recovered following the use of a  $\gamma$ -phosphate-linked ATP-agarose column, as used for other protein kinases [30]. Unexpectedly, little activity was eluted with MgATP, but higher levels were eluted with 1 M NaCl (Figure 1). This produced an approx. 500-fold purification of GCK activity, but the yield in the high-salt elution was only 0.7% of the original activity, and the eluted fractions contained multiple polypeptides. While a major 70 kDa polypeptide was present in the eluate as described previously [25], this did not co-distribute with GCK activity on subsequent chromatography on a Mono Q column (results not shown). An alternative purification strategy was therefore developed and optimized for rapid purification of GCK activity from milk.

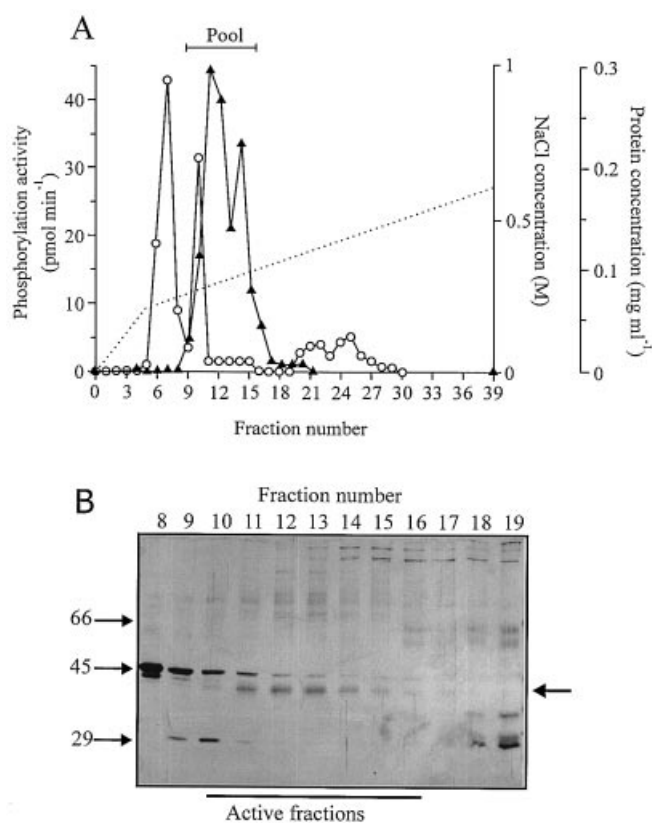


**Figure 2** Fractionation of GCK activity using phenyl-Sepharose and heparin-Sepharose chromatography

(A) Dialysed whey was loaded on to a phenyl-Sepharose column and bound proteins were eluted using a descending ammonium sulphate gradient (broken line). Fractions were collected and assayed for protein concentration (○) and GCK activity (■). The active fractions were pooled as indicated. (B) Pooled fractions from the phenyl-Sepharose column were applied on to a heparin-Sepharose column, and bound proteins were eluted with an ascending NaCl gradient (broken line). Fractions were collected and assayed for protein concentration (○) and GCK activity (■). The active fractions were pooled as indicated.

In the optimal purification protocol, dialysed whey was applied directly to a phenyl-Sepharose column, and GCK activity was eluted at the end of a decreasing ammonium sulphate gradient as a single peak (Figure 2A). Active fractions (fractions 20–23) were pooled and applied directly to a heparin-Sepharose column, from which GCK activity was eluted approximately midway in a 0–1.0 M NaCl gradient as a single peak (Figure 2B). Pooled active fractions (fractions 16–18) were dialysed and applied to a high-resolution ion-exchange Mono Q column. In initial experiments up to 85% of GCK activity was lost at this stage during dialysis. In order to reduce this loss, the dialysis membrane was pretreated to remove heavy metal ions; 10% glycerol was added to the dialysis buffer and dialysis was carried out for only 3 h. GCK activity was bound to the Mono Q column and eluted between 200 and 300 mM NaCl in two closely migrating peaks (Figure 3A). The presence of two peaks was seen in three separate runs of this purification protocol. The entire purification was carried out as rapidly as possible without freezing active fractions at any point, which otherwise resulted in a substantial loss of activity.

The purification of GCK from bovine milk as described above resulted in an 84000-fold purification, with a 46% yield of



**Figure 3** Partial purification of GCK activity using ion-exchange chromatography on Mono Q

(A) Active fractions from sequential phenyl-Sepharose and heparin-Sepharose chromatography were supplied to a Mono Q column, and bound proteins were eluted with an ascending NaCl gradient (broken line). Fractions were collected, assayed for protein concentration (○) and GCK activity (▲), and active fractions pooled as indicated. (B) SDS/PAGE analysis of fractions from Mono Q chromatography. Fractions were concentrated by precipitation with methanol at  $-20^{\circ}\text{C}$ , separated on SDS/10% PAGE, and stained with Coomassie Blue. Molecular-mass standards are indicated on the left. The polypeptide which co-distributed with GCK activity, and which was taken for peptide sequence analysis, is indicated by the arrow on the right.

enzyme activity (Table 1). In each of the last two steps of the purification the total activity increased. This is likely to be due to the removal of endogenous casein that competed with the exogenous peptide substrate. Examination of the polypeptide content of the final fractions from the Mono Q column by SDS/PAGE revealed the presence of several polypeptides in the active fractions (Figure 3B). Following either silver-staining (not

shown) or Coomassie Blue-staining (Figure 3B), only one polypeptide, migrating at approx. 40 kDa, appeared to co-distribute with GCK activity in all three purifications carried out. Six peptides derived from this polypeptide following proteolysis by trypsin were sequenced giving the sequences LAVYQAGA, FGLVEQGQSR, QQLEEVHA, LQAEAFQ, SWFEPLVED-MQR and VQLALRPS. All six sequences were found to be derived from bovine apolipoprotein E. This suggests that the GCK activity may still be a minor protein component in the Mono Q fractions, even following this substantial purification. At this stage, GCK activity was highly unstable, being quickly lost at  $4^{\circ}\text{C}$  or even in rapidly frozen samples, and so further purification was not possible.

## DISCUSSION

We have demonstrated here that soluble GCK activity can be detected in milk at significant levels of activity. These results suggest that GCK is secreted along with other milk proteins. It had not been previously suspected that GCK could be present in milk, since it was assumed to be an integral membrane protein of the Golgi. One possibility is that the GCK activity in milk is due to the release of an active luminal fragment following limited proteolysis of a transmembrane protein. This has been demonstrated to occur for galactosyltransferase [31,32] in mammary cells and also for sialyltransferase in other cell types [18]. The data on the solubility of GCK activity following removal of detergent could also be consistent with it being a soluble luminal enzyme. The specific activity of GCK detected in milk was severalfold lower than the specific activity detected in mammary Golgi fractions [13,27]. This is not surprising, as it would be likely that, for an enzyme with a specific Golgi function, mechanisms would exist to allow its retention in the Golgi. It is important, however, that the specific activity of GCK in milk was up to 6-fold higher than that assayed in Golgi fractions of other tissues such as brain and kidney [13], indicating that this was not a trivial level of enzyme activity. It is possible that some physiological function exists for the GCK in milk, for example in post-secretion phosphorylation of any non-phosphorylated casein that is secreted and stored within the lumen of mammary alveoli.

From the data on purification of GCK activity we can set an upper limit for the amount of enzyme secreted into milk. After correcting for loss of activity this would amount to only 0.001% of total milk protein. In all mammalian species the level of milk secretion is close to 1.67 ml/g of tissue in a 24 h period [33]. This would mean secretion of  $\leq 0.6 \mu\text{g}$  of GCK over this time (a relatively low level of protein). Without knowing more information about the relative turnover of the Golgi enzyme it cannot be determined further what proportion is likely to be

**Table 1** Purification of GCK activity from bovine milk

Samples from each of the purification steps were retained and assayed for GCK at the same time.

Purification step	Volume (ml)	Protein concn. (mg/ml)	Total activity (pmol/min)	Yield (%)	Specific activity (pmol/min per mg)	Fold purification
Milk	200	36.38	60 400	100	8.3	1
Whey	100	8.12	34 600	57.28	42.6	5.1
Phenyl-Sepharose	12	6.94	15 912	26.34	191.1	23.0
Heparin-Sepharose	9	0.17	25 380	42.02	16 494.1	1987.3
Mono Q	4	0.01	27 958	46.28	698 950	84 210.8

secreted from such an analysis. An instructive comparison can be made, however, with galactosyltransferase. As well as a clipped form being secreted [31], this is known to exist as a Golgi transmembrane enzyme [34], with a specific activity in Golgi membranes of 176 nmoles/min per mg of protein, and for the secreted form in milk of 2.3 nmoles/min per mg of protein [31,32] (a ratio of 76.5:1). The specific activities of GCK are 1433 [27] and 30.6 (the present paper) pmoles/min per mg of protein in Golgi and milk respectively, giving a ratio of 47.7:1. This is similar to that for galactosyltransferase, suggesting that secretion of only a small proportion of the GCK is likely to occur, as reported for the galactosyltransferase.

Despite its physiological significance in the phosphorylation of many diverse secretory proteins, GCK has never been characterized at a molecular level, although it is clearly distinct from the cytosolic enzymes CK1 and CK2. The purification strategy developed here relied on the selective phosphorylation of a synthetic peptide, based on a phosphorylation site in bovine  $\beta$ -casein, to probe specifically for GCK. Neither CK1 nor CK2 is able to effectively phosphorylate this peptide [27]. A third cytosolic CK has been identified from the lactating goat mammary gland [35]. This enzyme can phosphorylate casein *in vitro*, but its activity is inhibited by 5 mM  $Mn^{2+}$ , unlike the kinase activity purified in the present paper, which was assayed in the presence of 15 mM  $Mn^{2+}$ . In addition, any cells present in milk were removed by centrifugation and, therefore, it is unlikely that cytosolic kinases would be detectable in the present study.

The GCK activity purified from milk ran as a single activity during chromatography on phenyl- or heparin-Sepharese. During Mono Q chromatography, however, the activity routinely resolved as two closely migrating peaks. This might be suggestive of the existence of two closely related GCKs. Resolution of two activities was also found when freshly prepared whey fraction was immediately separated by ion-exchange chromatography (results not shown), arguing against the generation of two forms by limited proteolysis during the purification protocol. It is significant that previous work has indirectly suggested the existence of two GCKs in mammary tissue based on differing substrate specificities and cofactor requirements [36,37]. In addition, dissection of the secretory pathway in mouse lactating mammary epithelial cells using brefeldin A has suggested the presence of two GCK activities located in either *cis*- or *trans*-Golgi compartments with different substrate specificities [7]. Similar results have been found for phosphorylation of insulin-like growth factor-1 binding protein-1 in 3T3 cells [20]. In other studies with brefeldin A, which have examined single substrates, opposite conclusions were reached about the site of phosphorylation in *cis*/medial versus *trans*-Golgi compartments [14, 18,38]. Overall, these results suggest the possible existence of two related, but distinct, GCKs, with different localizations or sites of action within the Golgi.

The optimal purification protocol used here allowed enrichment of GCK to a high specific activity at high yield. Previous attempts at purification of GCK from mammary gland or liver achieved considerably lower degrees of purification [13,25], and it seems probable that the polypeptides suggested to be the GCK will instead have been contaminants. Indeed the 70 kDa protein isolated in one study was found by immunocytochemistry to be mammary-gland specific [25], a result inconsistent with our current knowledge of the widespread distribution of GCK activity. A more recent attempt achieved a 107-fold purification from liver Golgi, and from a similar purification identified a 170 kDa polypeptide from mammary gland as a potential catalytic subunit of GCK [13]. Using the peptide substrate as used here, these preparations had specific activities between 130-

2000-times lower than the most purified material obtained in the present study, casting doubt on this suggested identification of GCK. We were unable, despite substantial purification, to determine the protein composition of GCK from milk, as the only polypeptide co-purifying with GCK activity turned out to be apolipoprotein E. Other attempts to identify the active kinase subunits were made but were unsuccessful. For example, an examination of autophosphorylated polypeptides did not reveal a labelled polypeptide that co-purified with GCK activity. A recent similar attempt to use this approach to identify GCK identified endoplasmin instead of a kinase subunit [39]. Similarly, the use of the ATP analogue *p*-fluorosulphonylbenzoyl 5'-adenosine [13] did not convincingly detect a polypeptide co-purifying with GCK activity in our hands. We were also unable to detect binding of substrate ( $\beta$ -casein) to purified polypeptides.

The most purified fraction obtained in the present study had a specific activity of approx. 0.69  $\mu$ moles/min per mg of protein. This is lower than that of some purified kinases, such as mitogen-activated protein kinase [30], and substantially (100-fold) lower than the purified catalytic subunit of cAMP-dependent protein kinase (62  $\mu$ moles/min per mg of protein) [40]. It is possible, therefore, that one or more additional substantial purification steps would be required to purify the physiological GCK to homogeneity. Alternatively, other strategies, such as expression cloning, may be required before molecular characterization of this elusive enzyme and its potential multiple isoforms will be possible.

This work was supported by a Wellcome Trust Veterinary Clinical Training Award to J.S.D.

## REFERENCES

- Burgoyne, R. D. and Duncan, J. S. (1998) Secretion of milk proteins. *J. Mammary Gland Biol. Neoplasia* **3**, 275–286
- Mercier, J.-C. and Gaye, P. (1980) Study of secretory lactoproteins: primary structures of the signals and enzymatic processing. *Ann. N.Y. Acad. Sci.* **343**, 232–251
- West, D. W. (1986) Structure and function of the phosphorylated residues of casein. *J. Dairy Res.* **53**, 333–352
- Bingham, E. W., Farrell, H. M. and Basch, J. J. (1972) Phosphorylation of casein. Role of the Golgi apparatus. *J. Biol. Chem.* **247**, 8193–8194
- West, D. W. and Clegg, R. A. (1983) Casein kinase activity in rat mammary gland Golgi vesicles. Phosphorylation of endogenous caseins. *Eur. J. Biochem.* **137**, 215–220
- West, D. W. and Clegg, R. A. (1984) Casein kinase activity in rat mammary gland Golgi vesicles. Demonstration of latency and requirement for a transmembrane ATP carrier. *Biochem. J.* **219**, 181–187
- Turner, M. D., Handel, S. E., Wilde, C. J. and Burgoyne, R. D. (1993) Differential effect of brefeldin A on phosphorylation of the caseins in lactating mouse mammary epithelial cells. *J. Cell Sci.* **106**, 1221–1226
- Duncan, J. S. and Burgoyne, R. D. (1996) Characterization of the effects of  $Ca^{2+}$  depletion on the synthesis, phosphorylation and secretion of caseins in lactating mammary epithelial cells. *Biochem. J.* **317**, 487–493
- Bingham, E. W. and Farrell, H. M. (1974) Casein kinase from the Golgi apparatus of lactating mammary gland. *J. Biol. Chem.* **249**, 3647–3651
- Bingham, E. W. and Groves, M. L. (1979) Properties of casein kinase from lactating bovine mammary gland. *J. Biol. Chem.* **254**, 4510–4515
- Capasso, J. M., Keenan, T. W., Abejón, C. and Hirschberg, C. B. (1989) Mechanism of phosphorylation in the lumen of the Golgi apparatus. *J. Biol. Chem.* **264**, 5233–5240
- Allende, J. E. and Allende, C. C. (1995) Protein kinases. 4. Protein kinase CK2 – an enzyme with multiple substrates and a puzzling regulation. *FASEB J.* **9**, 313–323
- 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
- Rosa, P., Mantovani, S., Rosboch, R. and Huttner, W. B. (1992) Monensin and brefeldin A differentially affect the phosphorylation and sulfation of secretory proteins. *J. Biol. Chem.* **267**, 12227–12232

- 15 Watkinson, A., Young, J., Varro, A. and Dockray, G. J. (1989) The isolation and chemical characterisation of phosphorylated enkephalin-containing peptides from bovine adrenal medulla. *J. Biol. Chem.* **264**, 3061–3065
- 16 Dockray, G. J., Varro, A., Desmond, H., Young, J. and Gregory, H. (1987) Post-translational processing of the porcine gastrin precursor by phosphorylation of the COOH-terminal fragment. *J. Biol. Chem.* **262**, 8643–8647
- 17 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
- 18 Ma, J., Simonovic, M., Qian, R. and Colley, K. J. (1999) Sialyltransferase isoforms are phosphorylated in the cis-medial Golgi on serine and threonine residues in their luminal sequences. *J. Biol. Chem.* **274**, 8046–8052
- 19 Oetting, W. S., Tuazon, P. T., Traugh, P. T. and Walker, A. M. (1986) Phosphorylation of prolactin. *J. Biol. Chem.* **261**, 1649–1652
- 20 Peterkofsky, B., Gosiewska, A., Wilson, S. and Kim, Y.-R. (1998) Phosphorylation of rat insulin-like growth factor binding protein-1 does not affect its biological properties. *Arch. Biochem. Biophys.* **357**, 101–110
- 21 Bishop, L., Dimaline, R., Blackmore, C., Deavall, D., Dockray, G. J. and Varro, A. (1998) Modulation of the cleavage of the gastrin precursor by prohormone phosphorylation. *Gastroenterology* **115**, 1154–1162
- 22 Mercier, J.-C. (1981) Phosphorylation of caseins, present evidence for an amino acid triplet code posttranslationally recognised by specific kinases. *Biochimie* **63**, 1–17
- 23 Vegh, M. and Varro, A. (1994) Phosphorylation of human preprogastrin 93–101 by a Golgi membrane kinase from rat mammary gland. *Biochim. Biophys. Acta* **1205**, 49–53
- 24 Vegh, M. and Varro, A. (1997) Phosphorylation of gastrin-related peptides: physiological casein kinase like enzyme in Golgi membranes from bovine adrenal chromaffin cells and GH3 cells. *Regul. Pept.* **68**, 37–43
- 25 Moore, A., Boulton, A. P., Heid, H. W., Jarasch, E.-D. and Craig, R. K. (1985) Purification and tissue-specific expression of casein kinase from the lactating guinea-pig mammary gland. *Eur. J. Biochem.* **152**, 729–737
- 26 Pascall, J. C., Boulton, A. P. and Craig, R. K. (1981) Characterisation of a membrane-bound serine-specific casein kinase isolated from lactating guinea-pig mammary gland. *Eur. J. Biochem.* **119**, 91–99
- 27 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
- 28 Glass, D. B., Masaracchia, R. A., Feramisco, J. R. and Kemp, B. F. (1978) Isolation of phosphorylated peptides and proteins on ion exchange papers. *Anal. Biochem.* **87**, 566–575
- 29 Rosenfeld, J., Capdevielle, J., Guillemot, J.-C. and Ferrara, P. (1992) In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. *Anal. Biochem.* **203**, 173–179
- 30 Haystead, C. M. M., Gregory, P., Sturgill, T. W. and Haystead, T. A. J. (1993)  $\gamma$ -phosphate-linked ATP-sepharose for the affinity purification of protein kinases. *Eur. J. Biochem.* **214**, 459–467
- 31 Barker, R., Olsen, K. W., Shaper, J. H. and Hill, R. L. (1972) Agarose derivatives of uridine diphosphate and N-acetylglucosamine for the purification of a galactosyltransferase. *J. Biol. Chem.* **247**, 7135–7147
- 32 Smith, C. A. and Brew, K. (1979) Isolation and characterisation of a galactosyltransferase from Golgi membranes of lactating sheep mammary gland. *J. Biol. Chem.* **252**, 7234–7239
- 33 Mepham, T. B. (1983) *Biochemistry and Lactation*, Elsevier, Amsterdam
- 34 Russo, R. N., Shaper, N. L., Taatjes, D. J. and Shaper, J. H. (1992)  $\beta$ 1,4-galactosyltransferase: a short NH<sub>2</sub>-terminal fragment that includes the cytoplasmic and transmembrane domain is sufficient for Golgi retention. *J. Biol. Chem.* **267**, 9241–9247
- 35 Mitev, V. I., Angelov, I. V. and Marekov, L. N. (1992) Identification and purification of a novel serine/threonine messenger-independent growth-related protein kinase from lactating goat mammary gland. *Biochim. Biophys. Acta* **1117**, 90–96
- 36 Brooks, C. L. (1989) Two physiological substrate-specific casein kinases are present in the bovine mammary gland. *FEBS Lett.* **243**, 385–388
- 37 West, D. W. and Clegg, R. A. (1984) Polyamine stimulation of casein phosphorylation: differential effects on the three major rat caseins. *Biochem. Soc. Trans.* **12**, 1046–1047
- 38 Varro, A. and Dockray, G. J. (1993) Post-translational processing of progastrin: inhibition of cleavage, phosphorylation and sulphation by brefeldin A. *Biochem. J.* **295**, 813–819
- 39 Brunati, A. M., Contri, A., Muenchbach, M., James, P., Marin, O. and Pinna, L. A. (2000) GRP94 (endoplasmic) co-purifies with and is phosphorylated by Golgi apparatus casein kinase. *FEBS Lett.* **471**, 151–155
- 40 Slice, L. W. and Taylor, S. S. (1989) Expression of the catalytic subunit of cAMP-dependent protein kinase in *Escherichia coli*. *J. Biol. Chem.* **264**, 20940–20946

Received 16 March 2000/8 June 2000; accepted 27 June 2000