Stanniocalcin 1 and 2 are secreted as phosphoproteins from human fibrosarcoma cells

Derek A. JELLINEK*, Andy C. CHANG*, Martin R. LARSEN†, Xin WANG‡, Phillip J. ROBINSON‡ and Roger R. REDDEL*¹

*Cancer Research Unit, Children's Medical Research Institute, Locked Bag 23, Wentworthville 2145, NSW, Australia, †Protein Research Group, Department of Molecular Biology, Odense University, Odense DK-5230, Denmark, and ‡Cell Signalling Unit, Children's Medical Research Institute, 214 Hawkesbury Rd, Westmead 2145, NSW, Australia

Stanniocalcin 1 (STC1) and stanniocalcin 2 (STC2) are two recently identified mammalian peptide hormones. STC1 plays a role in calcium and phosphate homeostasis, while the role of STC2 is unknown. We examined a human fibrosarcoma cell line, HT1080, that has high steady-state STC1 and STC2 mRNA levels, to determine whether these proteins are secreted. Following incubation of HT1080 cells with ³²P, labelled STC1 and STC2 were found to be secreted into the medium. STC1 was phosphorylated *in vitro* by protein kinase C (PKC). *In vitro* and *in vivo* phosphorylation both occurred exclusively on serine and the phosphopeptide maps were similar, suggesting that PKC might be the *in vivo* kinase. STC2 was phosphorylated *in vitro* by casein kinase II (CK2), *in vitro* and *in vivo* phosphorylation were exclusively on serine and the phosphopeptide maps were indistinguishable. Phosphorylation of STC2 in intact cells resulted from the action of an ecto-protein kinase, since exogenous STC2

was phosphorylated by HT1080 cells and no phosphorylated STC2 was detectable inside the cells. The ectokinase activity was abolished by heparin and GTP could substitute for ATP as the phosphate donor, indicative of an ecto-CK2-like activity. The *in vitro* CK2 phosphorylation site was shown by matrix-assisted laser-desorption ionization–time-of-flight MS to be a single serine located between Ser-285 and Ser-298 in the C-terminal region of STC2. This is the first report of the secretion of STC1 or STC2 from mammalian cells. We conclude that these human fibrosarcoma cells express both STC1 and STC2 as secreted phosphoproteins *in vivo*, with STC2 being phosphorylated by an ecto-CK2-like enzyme.

Key words: ecto-protein kinase, protein phosphorylation, stanniocalcin 2.

INTRODUCTION

Stanniocalcin 1 (STC1) is a secreted, dimeric glycoprotein hormone which is a regulator of calcium and phosphate homeostasis. STC1 stimulates phosphate reabsorption in rat proximal tubules [1] and inhibits Ca²⁺ absorption in swine and rat intestine *in vitro* [1,2]. STC1 immunostaining and mRNA have been detected in many tissues, including kidneys [3], ovaries [4] and neurons [5]. STC1 mRNA has also been detected in chondrocytes and osteoblasts, thereby implicating STC1 in endochondral and membrane bone formation [6]. This ubiquitous expression suggests that mammalian STC1 may have acquired autocrine or paracrine roles, in addition to the classical endocrine function observed with the fish homologue, STC [7]. STC was reported originally to play a major role in calcium and phosphate homeostasis in fish with bony skeletons [8].

Stanniocalcin 2 (STC2) is a recently reported STC1 homologue that shares ≈ 34% identity with STC1 [9]. STC2 also appears to be widely expressed in mammalian tissues, including pancreas [10,11], skeletal muscle [12] and small intestine [9]. The function of STC2 has yet to be fully clarified, but is also likely to relate to calcium and phosphate metabolism. One study, however, demonstrated that the effects of STC2 on phosphate transport in opossum kidney cells are unlike those of STC1. STC2 inhibited phosphate reabsorption by reducing the expression of a renal Na–P_i-4 co-transporter [12].

A variety of other peptide hormones are secreted as phosphoproteins. These include adrenocorticotropin [13], human chori-

onic gonadotropin [14], basic fibroblast growth factor [15], atrial natriuretic peptide [16,17], progastrin [18], growth hormone [19] and prolactin [20–22]. Some of these hormones are substrates for intracellular as well as extracellular protein kinases. Atrial natriuretic peptide and basic fibroblast growth factor have been shown to be phosphorylated extracellularly by an ecto-cAMP-dependent protein kinase, ecto-PKA, located on the external surface of cells [15,16].

In situ hybridization and immunohistochemical staining show the presence of STC1 in a variety of tissues. Therefore, our aim was to determine if endogenous STC1 and STC2 are secreted from mammalian cells. Since a variety of peptide hormones are secreted as phosphoproteins, we also examined the phosphorylation status of secreted STC1 and STC2.

EXPERIMENTAL

Cells, recombinant protein and antibodies

HT1080 cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The cDNAs encoding STC1 and STC2 were PCR-amplified with primers containing restriction-enzyme sites for cloning into the baculovirus expression vector pFastBac1 (Life Technologies, Victoria, Australia). STC1 also contained the epitope tag MRGSH₆ at the C-terminus to aid in its purification by metal-affinity chromatography. Recombinant STC1 and STC2 were purified from

Abbreviations used: STC1, stanniocalcin 1; STC2, stanniocalcin 2; PKC, protein kinase C; CK2, casein kinase II; MALDI-TOF, matrix-assisted laser-desorption ionization–time of flight; PKA, cAMP-dependent protein kinase; CaMK-II, calmodulin-dependent protein kinase II; PKG, protein kinase G; MARCKS, myristoylated alanine-rich C-kinase substrate; cdc2 kinase, p34^{cdc2}/cyclin B; Endo-Lys-C, endoprotease Lys-C.

¹ To whom correspondence should be addressed (e-mail redde@cmri.usyd.edu.au).

the medium of baculovirus-infected Sf9 (*Spodoptera frugiperda*) cells propagated in SF900II medium (Life Technologies). Rabbit polyclonal and mouse monoclonal STC1 antibodies (a generous gift from Dr Yasuhiro Furuichi, AGENE Research Institute, Kanagawa, Japan) were made using recombinant STC1 expressed in *Escherichia coli* as the antigen. The rabbit polyclonal antibodies were immunoaffinity purified. Polyclonal anti-STC2 antibodies were made in sheep using peptides corresponding to three different regions of the STC2 molecule not represented in STC1 (peptide A, TDATNPPEGPQDRSSQKKG; peptide B, CSK-SHPNAHARGRVGGLGAQ; peptide C, CSGSEWEDEQS-EYSIDRR). Antiserum was purified on a Protein G-Sepharose column (Amersham Pharmacia Biotech, Uppsala, Sweden) followed by affinity purification on a streptavidin column (Pierce, Rockford, IL, U.S.A.) to which biotinylated peptide A was coupled (peptide A was determined by ELISA to be the most immunogenic of the three peptides).

³²P Labelling of cells and Western blotting

HT1080 cells were plated at 9×10^5 cells/well in 6-well plates (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) and grown overnight. The following day the cells were rinsed three times with PBS, then incubated with 200 μ Ci/ml ³²P (9000 Ci/mmol; Amersham, Victoria, Australia) in fresh Dulbecco's modified Eagle's medium without fetal bovine serum for 24 h at 37 °C. Cell lysates were extracted in RIPA buffer (1 % Nonidet P40, 1 % sodium deoxycholate, 0.0125 M sodium phosphate, pH 7.2, 2 mM EDTA, 0.2 mM sodium vanadate, 50 mM sodium fluoride, 100 units/ml aprotinin, 10 μ g/ml leupeptin and 1 μ g/ml pepstatin A) at 4 °C for 5 min, followed by centrifugation at 4 °C for 25 min at 14000 g. Secreted STC1 and STC2 were immunoprecipitated from HT1080-conditioned medium and cellular lysates by adding 1–10 μ g of purified polyclonal STC1 or polyclonal STC2 antibodies, respectively, without pre-clearing. The samples were then agitated slowly overnight at 4 °C. Either Protein A-agarose or Protein G-agarose (Pierce) was added to each tube for a further 3 h at 4 °C. Samples were centrifuged and the beads were washed seven times with RIPA buffer before dissolving in a 3 $\times$ concentrate of SDS/PAGE sample buffer (0.5 M Tris/HCl, pH 6.8, 28 % glycerol, 6 % SDS, 6 mM EGTA, 25 mM β -mercaptoethanol and 0.7 mg/ml Bromophenol Blue) and heated to 95 °C for 3 min. The samples analysed for carbohydrate moieties were processed differently. After washing with RIPA buffer, the samples were resuspended in 1 % SDS, boiled for 2 min, then 20 mM sodium phosphate, pH 7.2, 10 mM Na₃, 50 mM Nonidet P40 and 0.5 % *n*-octylglucoside were added and the samples boiled for an additional 2 min. Once cooled, 0.4 unit of N-glycosidase F (Boehringer Mannheim, Mannheim, Germany) was added and the samples incubated at 37 °C for 16 h. All samples were centrifuged and analysed by SDS/PAGE using 10 or 12 % polyacrylamide gels, followed by transferring to a PVDF membrane (Millipore, Bedford, MA, U.S.A.). Blots were exposed to X-ray film to detect phosphoproteins and were then processed for immunodetection of STC1 or STC2. Membranes were blocked in 3 % BSA in TBS/Tween (10 mM Tris/HCl, pH 7.4, 150 mM NaCl and 0.1 % Tween-20) for 2 h, probed with either monoclonal STC1 or polyclonal STC2 antibodies in 1 % polyvinylpyrrolidone (PVP-40) in TBS/Tween for 1 h at room temperature, washed in PBS five times for 5 min each, then probed with horseradish peroxidase-conjugated secondary antibody (Dako Corporation, Carpinteria, CA, U.S.A.) in the same buffer for 1 h at room temperature. The blots were further washed with PBS five times

for 5 min each and detected by chemiluminescence (Pierce Super Signal) according to the manufacturer's instructions.

Ecto-protein kinase assay

Confluent monolayers of HT1080 cells in 0.32-cm² wells were preincubated in 0.02 ml of protein kinase buffer (20 mM Hepes, pH 7.4, 145 mM NaCl, 1 mM CaCl₂ and 1 mM MgCl₂) containing test substances for 10 min at 23 °C. Then, 5 μ Ci of [γ -³²P]ATP or [γ -³²P]GTP, 0.2 mM Na₃VO₄ and 1 μ g of recombinant STC1, STC2 or 50 μ g of dephosphorylated casein (Sigma, Castle Hill, New South Wales, Australia) were added and the reaction was incubated for 15 min at 37 °C. Phosphorylation was terminated by removing the reaction mixture from the cells and adding 0.02 ml of 3 $\times$ SDS sample buffer. Samples were heated at 95 °C for 3 min, cooled and loaded on to SDS/polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue R-250 [1.5 mg/ml, 23 % (w/v) trichloroacetic acid] for 30 min, destained and dried. Conditions for detecting released ecto-kinase activity were similar to those described above, with the exception of the substrate being added to the cells for 15 min at 23 °C and then the conditioned medium being transferred to a tube containing [γ -³²P]ATP or [γ -³²P]GTP for 15 min at 37 °C. Radiolabelled bands were detected and quantified on a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Protein kinase assay

Phosphorylation of recombinant STC1 and STC2 was carried out with 30 mM Tris/HCl (pH 7.4), 1 mM EGTA, 1 mM MgSO₄, 40 μ M ATP, 0.125 μ Ci/ml [γ -³²P]ATP (3000 Ci/mmol, Amersham) and 0.05 % Tween 80 in a final volume of 80 μ l. The added protein kinases included: protein kinase C (PKC) [23] with 1.2 mM CaCl₂ (generating 200 μ M free Ca²⁺), 40 μ g/ml L-phosphatidylserine and 4 μ g/ml 1,2-diolein; or calmodulin-dependent protein kinase II (CaMPK-II) with 10 μ g/ml calmodulin (Calbiochem, Alexandria, New South Wales, Australia) and 1.2 mM CaCl₂; or protein kinase G (PKG) [23] with 10 μ M cGMP. A peptide derived from the Walsh inhibitor of PKA [23], PKI, at 0.01 mg/ml was included in the assays for PKG and PKC to prevent cross-activation by cGMP of any PKA potentially present in the samples. Human recombinant p34^{cdc2}/cyclin B (cdc2 kinase) and casein kinase II (CK2) were purchased from Calbiochem. Upon prewarming samples to 37 °C for 5 min, reactions were initiated by addition of 500 ng of recombinant STC1 or STC2, followed by the purified kinase (20–40 ng/reaction). After 5 min the reactions were terminated by the addition of 50 μ l of 3 $\times$ SDS sample buffer and rapid freezing on dry ice [24]. Phosphorylated STC was resolved using a 7.5–15 % acrylamide-gradient SDS gel (20 cm², Protean II system, Bio-Rad, Regents Park, New South Wales, Australia).

Determination of stoichiometry and substrate kinetics utilized similar conditions as described above. However, a time course to determine P_i saturation at a fixed protein concentration was utilized for stoichiometric analyses whereas kinetic analyses employed a 10-min incubation at 37 °C along with a broad protein-concentration range. Phosphorylated species were excised from dried gels, counted in a β -counter, and stoichiometry and kinetics were determined. Kinetic constants K_m and V_{max} were derived with the PC program Enzfitter (written by Robin Leatherbarrow, from Sigma). Myristoylated alanine-rich C-kinase substrate (MARCKS) was isolated and purified from rats as described previously [25].

Phosphoamino acid analysis, phosphopeptide mapping and matrix-assisted laser-desorption ionization–time-of-flight (MALDI-TOF) MS

In vitro- or *in vivo*-phosphorylated STC1 and STC2 were resolved by SDS/PAGE (12% gel) and transferred on to a PVDF membrane. The bands were excised using the autoradiograph as a template and hydrolysed in 6 M HCl at 110 °C for 60 min. The samples were then dried in a Speed-Vac Concentrator (Savant, Farmingdale, NY, U.S.A.), dissolved in pyridine/acetic acid/water (5:50:945, by vol.), pH 3.5, containing unlabelled phosphotyrosine, phosphothreonine and phosphoserine (Sigma) each at 1 mg/ml and the phosphoamino acids were separated by thin-layer electrophoresis as described previously [26]. Phosphopeptide maps using V8 protease (*Staphylococcus aureus*; Sigma) were performed on recombinant and native STC1 and STC2 excised from dried gels, digested and peptides were separated by SDS/PAGE (15% gel) as described previously [27].

For MS, 50 µg of *in vitro*-phosphorylated STC2 was reduced and carboramidomethylated *in situ* before being digested with 5 µg of endoproteinase Lys-C (Endo-Lys-C, *Lysobacter enzymogenes*; Boehringer Mannheim) for 48 h at 37 °C. The sample was extracted in 1% trifluoroacetic acid/40% acetonitrile overnight, dried, resuspended in 200 µl of water and loaded directly on to an HPLC micro reversed-phase chromatography (µRPC) C₂/C₁₈ column (Amersham Pharmacia Biotech). Labelled peptides were detected by Cerenkov counting and the fraction containing the highest radioactivity was micro-purified on a Poros R3 column [28]. The sample was analysed by MALDI-TOF MS in linear delayed extraction (LDE) mode (Perkin Elmer-PerSeptive Biosystems Voyager DE-STR, Melbourne, Australia). Alkaline phosphatase (Boehringer Mannheim)-treated samples were digested on target with 0.25 unit of alkaline phosphatase in 2 µl of 0.1 M NH₄HCO₃, pH 7.8 [29].

RESULTS

Analysis of ³²P-labelled STC1 and STC2

To study the secretion of the mammalian STCs, it was first necessary to find a cell line that secreted either STC1 or STC2, as no cell lines have been reported to secrete these hormones. The human fibrosarcoma cell line, HT1080, containing a high level of steady-state STC1 mRNA [7], was found to contain STC2 mRNA (results not shown) and was therefore used in the present study. In addition, the secretion of STC1 and STC2 observed from HT1080 cells was not due to leakage from cellular debris. This was verified by dye exclusion, which showed a > 96% cell viability under experimental conditions (results not shown).

To demonstrate storage and secretion of the STCs, Western-blot analyses were performed on cell lysates and conditioned media from HT1080 cells using specific antibodies to STC1 and STC2 (Figure 1). STC1 was produced in high abundance in the cells, detected as a 31-kDa doublet and a minor 33-kDa band (Figure 1A, lane 3). Smaller amounts of STC1 were secreted from these cells as a larger 35-kDa form and a 31-kDa form (Figure 1A, lane 4). The multiple size forms detected were largely accounted for by glycosylation, as digestion of cellular lysate and conditioned media with N-glycosidase F resulted in an increased mobility for all bands (Figure 1C).

The same cells produced and secreted STC2; however, the pattern was markedly different from STC1. STC2 was undetectable in the cells by Western blot (Figure 1B, lane 3), but significant levels of the 32-kDa protein were detected in a secreted form (Figure 1B, lane 4). This suggests that, unlike

Figure 1 Immunoprecipitation of STC1 or STC2 from ³²P-labelled cellular lysates or conditioned medium

Confluent HT1080 cells were labelled for 24 h with ³²P and the cell lysate (lanes 1 and 3) or conditioned medium (lanes 2 and 4) were immunoprecipitated with either (A) polyclonal STC1-specific or (B) polyclonal STC2-specific antibodies. Radiolabelled proteins were separated by SDS/PAGE, followed by transfer to PVDF membranes and analysed by autoradiography and Western blotting. Blots were probed with either monoclonal STC1 or polyclonal STC2 antibodies, respectively. (C) A Western blot containing polyclonal STC1 immunoprecipitates of cellular lysate (lanes 1 and 2) and conditioned medium (lanes 3 and 4) which were either treated (lanes 2 and 4) or untreated (lanes 1 and 3) with N-glycosidase F. Blots were probed with monoclonal STC1 antibody and the positions of antibody heavy (Ig_H) and light (Ig_L) chains are shown. Results are representative of four separate experiments.

STC1, newly synthesized STC2 is not stored in the cells, but undergoes constitutive secretion.

To determine if the secreted hormones might be phosphorylated, HT1080 cells were labelled with ³²P and either STC1 or STC2 was immunoprecipitated. Autoradiography detected STC1 as a 35-kDa phosphoprotein (Figure 1A, lane 2). Phosphorylation of STC1 was only detected in the conditioned medium and the band co-migrated in size with the heavily glycosylated form of STC1 (Figure 1A, lane 4). The majority of the cellular STC1 (Figure 1A, lane 3) did not co-migrate with any

Figure 2 Extracellular phosphorylation of recombinant STC2 and dephosphorylated casein by ecto-protein kinases associated with HT1080 cells

Intact HT1080 cells were labelled for 15 min with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (A) or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (B) with (lanes 3–7) or without (lanes 2) 1 μg of recombinant STC2. The recombinant STC2 preparation showed no intrinsic protein kinase activity (lanes 1). Some samples were pretreated for 10 min with 10 μM cAMP (lanes 4), 10 μM cGMP (lanes 5), 1 μM PMA (lanes 6) or 5 μg of heparin (lanes 7). (C) HT1080 cells were incubated with 50 μg of dephosphorylated casein with (lanes 5 and 6) or without (lanes 1–4) 50 μg of heparin. In lanes 1, 3 and 5, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was utilized as the phosphate donor, whereas in lanes 2, 4 and 6 $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ was used. No intrinsic protein kinase activity was present in the dephosphorylated casein preparation (C and D, lanes 1 and 2). (D) To determine released ecto-kinase activity, HT1080 cells were treated for 15 min with 50 μg of dephosphorylated casein and the conditioned medium collected by aspiration. This medium was then tested for released ecto-protein kinase activity by adding either $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (lane 3) or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (lane 4). Results are representative of three experiments.

Figure 3 Phosphorylation of STC1 and STC2 by purified protein kinases *in vitro*

Recombinant STC1 (A) or STC2 (B) were phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of various serine/threonine protein kinases as indicated. The proteins were separated by SDS/PAGE on a 7.5–15% acrylamide gel, fixed, dried and exposed to a PhosphorImager screen. STC1 results were reproduced in three experiments. cdc2, p34^{cdc2}/cyclin B.

Table 1 Kinetics of phosphorylation of STC1 and MARCKS by PKC as well as of STC2 and dephosphorylated casein by CK2

The apparent kinetic constants were determined from Lineweaver–Burke plots. The substrate specificity was determined by calculating the $V_{\max}/K_m$ ratio for each protein kinase.

Substrate	K_m (μ M)	$V_{\max}$ (nmol/mg per min)	$V_{\max}/K_m$
STC1	0.61 ± 0.34	1.94 ± 0.45	3.18
MARCKS	9.52 ± 4.17	4.99 ± 0.95	0.52
STC2	1.96 ± 0.035	8.69 ± 0.51	4.43
Dephosphorylated casein	4.98 ± 0.77	40.2 ± 3.1	8.07

Figure 4 Phosphoamino acid analysis of *in vivo*- and *in vitro*-phosphorylated human STC1 and STC2

Human STCs were either immunoprecipitated with specific anti-STC1 antibodies (**A**, lane 1) or specific anti-STC2 antibodies (**B**, lane 1) from ^{32} P-labelled HT1080-conditioned medium. Recombinant STCs were phosphorylated with PKC (**A**, lane 2) or CK2 (**B**, lane 2) using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as the phosphate donor. The labelled proteins were resolved by SDS/PAGE on a 12% gel and transferred on to PVDF membranes. The resultant phosphoproteins were then subjected to phosphoamino acid analysis. An autoradiograph is shown with the positions of phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr) standards on the left. Results are representative of two separate experiments.

^{32} P-labelled bands (Figure 1A, lane 1), although a small amount of the 33-kDa medium glycosylated form might correlate with a ^{32} P-labelled band. Therefore, mainly the secreted form of STC1 was phosphorylated. This phosphorylation may occur as a late event in post-translational processing, with STC1 possibly being phosphorylated during or soon after secretion from the cell.

When polyclonal STC2-specific antibodies were used to immunoprecipitate from labelled HT1080-conditioned medium, a similar result was obtained. No cellular phosphorylation of STC2 was detected (Figure 1B, lane 1), but a major secreted phosphoprotein was immunoprecipitated (Figure 1B, lane 2) that co-migrated with immunoreactive STC2 (Figure 1B, lane 4). The phosphorylated STC2 ran as a doublet, with a minor

Figure 5 Comparison of *in vivo* and *in vitro* phosphorylation sites

Phosphopeptide mapping with V8 protease of either STC1 (lanes 1 and 2) or STC2 (lanes 3 and 4). The proteins were phosphorylated *in vitro* by PKC or CK2 (lanes 1 and 3), as indicated above the panel. The proteins were also immunoprecipitated from HT1080-conditioned medium after labelling with ^{32} P (lanes 2 and 4) as described in the legend for Figure 1, and visualized by autoradiography. Results are representative of two separate experiments.

amount of a larger-molecular-mass species, that may have been glycosylated.

Phosphorylation of exogenous STC2 by ecto-protein kinases

Since no phosphorylated STC1 or STC2 was detectable in the cellular lysates of labelled HT1080 cells, it seemed likely that the proteins were being phosphorylated during or after secretion. To determine whether an ecto-protein kinase might phosphorylate STC1 or STC2, HT1080 cells were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and exogenous recombinant STC was added. Ecto-protein kinase activity was only detectable using STC2 as a substrate (Figure 2). No intrinsic protein kinase activity was detected in STC2 preparations (Figure 2A, lane 1), but numerous substrates for ecto-protein kinases associated with HT1080 cells were detected (Figure 2A, lane 2). Exogenous STC2 inhibited the phosphorylation of some cell-surface proteins (Figure 2A, compare lane 2 with lanes 3–6), suggesting that they may be substrates for the same enzyme. Protein kinase activators cAMP, cGMP and PMA had no effect on STC2 phosphorylation (Figure 2A, lanes 3–6). The ecto-protein kinase was strongly inhibited by heparin (Figure 2A, lane 7). The ecto-protein kinase activity was able to utilize either ATP or GTP equally as phosphate donors (Figures 2A and 2B). These are two major characteristics of CK2 [30]. To further confirm the existence of

Figure 6 MALDI-TOF analysis of recombinant STC2 phosphorylated by CK2

STC2 (50 μ g) was phosphorylated *in vitro* by CK2 followed by digestion with Endo-Lys-C. The digest was purified on a C_2/C_{18} column and the radioactivity of each fraction was detected by Cerenkov counting. The fraction containing the highest amount of radioactivity was analysed further by MALDI-TOF before (A) and after (B) treatment with alkaline phosphatase (AP). The spectrum shows a number of phosphorylated masses. The largest of these, 4058 and 3901 Da, correspond to STC2_{266–302} or STC2_{266–301} (A). Upon alkaline phosphatase treatment (B), these peptides lose 80 Da, representing one phosphate group. Almost all other masses shown result from further digestion of the 4058-Da peptide by a contaminating peptidase and show a loss of 80 Da upon alkaline phosphatase treatment (arrows). This consecutive loss of amino acid residues allowed their identification, as shown by the amino acid letters between the peaks.

an ecto-CK2 kinase, exogenous dephosphorylated casein was substituted for STC2 and was also shown to be phosphorylated by the ecto-protein kinase (Figure 2C). In addition, protein kinase activity was released from the surface of intact HT1080 cells upon treatment with dephosphorylated casein (Figure 2D), consistent with substrate-induced ecto-CK2 kinase release from the surface of other cell types [31]. These results together indicate that the ecto-protein kinase has the characteristics of CK2.

In vitro phosphorylation

To determine which protein kinase(s) phosphorylate STC1 and STC2 *in vitro*, we screened a variety of purified protein kinases. STC1 was a better substrate for PKC than for PKG, and was effectively phosphorylated by four other kinases: the catalytic subunit of PKA, CaMPK-II, CK2 and cdc2 kinase (Figure 3A). In contrast, STC2 was a better substrate for CK2 than for all other kinases tested (Figure 3B).

The stoichiometry as well as the kinetics of phosphorylation for both STC1 by PKC and STC2 by CK2 were then determined. Using either STC1 or STC2, the calculated stoichiometry was 0.1 mol of P/mol of protein. The kinetics (K_m and V_{max}) of phosphorylation of STC1 by PKC and STC2 by CK2 are summarized and compared with known substrates in Table 1. When the ratios of V_{max}/K_m for both STC1 and STC2 are compared with the calculated V_{max}/K_m ratio for the phosphorylation of MARCKS by PKC or the phosphorylation of

dephosphorylated casein by CK2, it is clearly evident that both STC1 and STC2 are excellent substrates for PKC and CK2, respectively.

Correlation of *in vitro* and *in vivo* phosphorylation

We next determined if the *in vitro* phosphorylation of STC1 and STC2 by PKC and CK2, respectively, was consistent with these protein kinases playing a role in the phosphorylation of the proteins secreted from HT1080 cells. *In vitro* and *in vivo* phosphorylation were compared to determine whether the same amino acids were labelled and whether the proteins were phosphorylated in the same region of the molecule. Phosphoamino acid analysis revealed that both *in vitro*- and *in vivo*-labelled STC1 (Figure 4A) and STC2 (Figure 4B) were labelled exclusively on serine residues. This is consistent with PKC and CK2 being the *in vivo* protein kinases responsible for STC1 and STC2 phosphorylation, respectively. This also excludes a role of any protein tyrosine kinase. To further correlate *in vitro* and *in vivo* phosphorylation, V8 phosphopeptide mapping was performed. The phosphopeptide map of STC1 revealed that the phosphorylation pattern observed *in vivo*, upon immunoprecipitating labelled STC1 from HT1080-conditioned medium, was strikingly similar to that generated *in vitro* by PKC (Figure 5, lanes 1 and 2). The phosphopeptide map of STC2 revealed that the site (or sites) phosphorylated by CK2 *in vitro* was also similar to those phosphorylated by intact HT1080 cells (Figure 5, lanes 3 and 4).

This supports the possibility that the kinases identified as being most active *in vitro* phosphorylate the same site as the *in vivo* protein kinases.

Phosphorylation site in STC2

To determine the phosphorylation site for CK2 in STC2, MALDI-TOF MS was used (Figure 6). A single phosphorylation site was detected which resided in one of the five serines between Ser-285 and Ser-298. STC2 was phosphorylated *in vitro* by CK2 and run on SDS/PAGE. Using autoradiography, the labelled band was excised and digested with Endo-Lys-C for analysis of peptide mass. A number of phosphorylated masses were detected (Figure 6A). The largest two were 4058 and 3901 Da and corresponded to the C-terminus of STC2 at STC2_{266–302} (with the sequence SHPNAHARGRVGGLGAQGPGSGSSEWEDEQSEYSDIRR) and STC2_{266–301}, respectively. Phosphorylation was found to occur on a single residue, as dephosphorylation with alkaline phosphatase reduced the mass of both peptides by 80 Da (Figure 6B). Fortunately, further digestion of STC2 occurred, possibly due to a contaminating peptidase. This yielded a variety of truncated fragments of masses 3622, 3414, 3343, 3187, 3130 and 2976 Da, corresponding to the sequential removal of the N-terminal dipeptide AH followed by the amino acids A, R, G and R respectively. This sequence, AHARGR, corresponds to STC2_{270–275}. Every peptide lost 80 Da when dephosphorylated (Figure 6B). This supports the conclusion that a single phosphorylation site exists within the C-terminal region of STC2, between Ser-285 and Ser-298 (SGSSEWEDEQSEYS). Of the five serines in this region, Ser-288 (shown in bold) precisely matches the known consensus sequence motif for a CK2 phosphorylation site [32].

DISCUSSION

We have shown that the human fibrosarcoma cell line, HT1080, secretes both STC1 and STC2 as phosphorylated proteins. This is the first report of any cell line secreting either hormone. It is also particularly interesting that this cell line secretes both. The post-translational processing of the two proteins, however, appears to be regulated differently. STC1 is produced intracellularly in large amounts in lightly glycosylated forms which were not phosphorylated. The secreted form, on the other hand, is not as abundant and is highly glycosylated and phosphorylated. This would suggest that the secretion of STC1 from the cells is regulated, with the lightly glycosylated, unphosphorylated form being stored until arrival of a required external stimulus. In contrast, STC2 is produced in low amounts in the cell, being undetectable by Western blots, and the secreted form is phosphorylated. The low level of intracellular STC2 precursors indicates that the processing and secretion is constitutive in these cells. Thus the regulation of STC1 and STC2 secretion from the same cell are distinct processes, possibly reflecting different biological roles for each protein.

STC2 was shown to be a substrate for an ecto-protein kinase associated with HT1080 cells. No intracellular STC2 phosphorylation was detected and the secreted protein was phosphorylated. The STC2 kinase was shown to be ecto-CK2 by five criteria: heparin inhibition, cyclic nucleotide independence, the ability to equally use ATP or GTP as the phosphate donor, the ability to phosphorylate exogenous casein and shedding of the activity from the cell surface. The ability of the ecto-CK2 protein kinase to be shed from the cell surface is a unique feature and has been shown by the addition of exogenous substrates [31]. In addition, STC2 was phosphorylated *in vitro* by CK2 more strongly than by

a variety of other protein kinases tested. Phosphoamino acid analysis of *in vitro*-phosphorylated STC2 by CK2, as well as *in vivo*-phosphorylated STC2, indicated that only serine residues are phosphorylated. Comparison of the phosphopeptide maps of *in vitro*- and *in vivo*-phosphorylated STC2 show good correlation. This suggests that CK2 may be the *in vivo* kinase responsible for STC2 phosphorylation. MALDI-TOF MS revealed that the *in vitro* CK2 phosphorylation site is contained within a C-terminal region, between Ser-285 and Ser-298 (SGSSEWEDEQSEYS). Based on consensus sequence analysis for known protein kinases, Ser-288 (shown in bold) is the best candidate for the site for CK2 phosphorylation among the five serine residues in this region [30]. CK2 prefers to phosphorylate serine or threonine in the context of acidic amino acids on the C-terminal side (underlined). A glutamic or aspartic acid three amino acids C-terminal to the phosphorylation site is known to be essential, while further acidic residues contribute to a high V_{max} . CK2 also supports hierarchical phosphorylation, so that phosphorylation of Ser-288 would potentially make Ser-285 a CK2 substrate. This is unlikely to be the case for STC2, however, as only a single phosphate was detected in all peptides derived from the Endo-Lys-C digest.

The mechanism of STC1 phosphorylation in HT1080 cells was not unequivocally demonstrated. No clear evidence of ecto-mediated kinase activity was found when using STC1 as a substrate. Therefore, either the phosphorylation occurred intracellularly or the ecto-protein kinase assay was insufficiently sensitive. It is likely that an ecto-protein kinase plays a role in STC1 phosphorylation for two reasons. First, no cellular phosphorylated STC1 was detected. Secondly, the secreted form that was phosphorylated was also highly glycosylated and no highly glycosylated form was found in the cells. This suggests that this form of STC1 is destined for rapid secretion, while the major forms are stored. Ecto-PKC has been well characterized in a number of cell types [33–37] and phosphorylates a variety of proteins. Phosphorylation of peptide hormones by ecto-PKC has not previously been found. STC1 was, however, an *in vitro* substrate for PKC. Phosphoamino acid analysis of STC1 immunoprecipitated from HT1080 cells metabolically labelled with radiolabelled phosphate or phosphorylated *in vitro* by PKC indicated that STC1 is phosphorylated exclusively on serine residue(s) in both contexts. Further correlation between *in vivo*- and *in vitro*-phosphorylated STC1 was observed when comparing the phosphopeptide maps, which were indistinguishable. Taken together, these data indicate that PKC is the best candidate for STC1 phosphorylation *in vivo*.

Ecto-phosphorylation may play an important functional role for STC1 and STC2, as judged by its importance in regulation of other secreted phosphoproteins. Ecto-protein kinases, including ecto-PKA [16,38], ecto-PKC [35,39], ecto-CK2 [40,41] and other unidentified protein kinases, have been observed on the outer surfaces of many cells. CK2 is perhaps the best-characterized ecto-protein kinase [31,42] and is found on the surface of HeLa cells [40], lymphocytes [43] and some bone cells [41]. In intact lymphocytes, ecto-CK2 plays a key role in cytotoxicity, as inhibition of ecto-CK2 abolishes target cell death [43]. Ecto-CK2 may also play a role in platelet activation [44] and has been shown to stimulate neurotransmitter uptake in neuronal cells [45]. It was positively identified as CK2 by Western blotting and by MALDI-TOF MS analysis of tryptic peptides [31]. Other suggested substrates for ecto-CK2 include fibrinogen [46], blood coagulation factors Va and VIII [47], the bone glycoproteins osteopontin and sialoprotein [41] and a variety of unidentified cell-surface proteins [31]. STC2 is the first peptide hormone found to be a substrate for ecto-CK2 protein kinase.

The biological role of STC1 and STC2 phosphorylation has yet to be determined; however, the addition of one or more phosphate groups to other peptide hormones have been found to regulate hormone processing, structure and biological activity. During the processing of progastrin, phosphorylation determines prohormone cleavage rates and controls production of alternative active products [18]. Human chorionic gonadotropin, on the other hand, is phosphorylated both in cells and after secretion, this being regulated by phorbol esters and by dimers of α and β human chorionic gonadotropin [14]. Phosphorylation can change the conformation and subsequently affect hormone function by disrupting local ionic or steric interactions. Phosphorylation of prolactin causes unique structural changes determined by absorption and UV fluorescence spectroscopy [20,21]. This reduces its receptor binding and is restored by dephosphorylation [22]. The phosphorylation of basic fibroblast growth factor in its receptor-binding domain by PKA increases its affinity for the fibroblast-growth-factor receptor [48]. The conformational change associated with phosphorylation may help increase the protein's half-life, as seen in fibrinogen digestion by plasmin [49]. The phosphorylation of growth hormone at Ser-90 severely reduces its biological activity [21]. This impairment is mimicked by substituting glutamic acid for Ser-90 [21]. *In vitro* studies of fibrinogen show that phosphorylation increases fibre thickness in fibrin clots [50].

In summary, this study demonstrates for the first time that STC1 and STC2 are secreted phosphoproteins as well as *in vitro* substrates for PKC and CK2 respectively. The *in vitro* CK2 phosphorylation site of STC2 was shown by MALDI-TOF MS to be a single serine located between Ser-285 and Ser-298 at the C-terminus. In addition, STC2 is able to be phosphorylated by an ecto-protein kinase with characteristics of CK2. The significance of phosphorylation observed for other secreted hormones suggests that the phosphorylation of the human STCs may be an important means of regulating their biological activity in calcium and phosphate homeostasis.

We thank Yasuhiro Furuichi for the STC1 antibodies, Valentina Valova for help with the mass analysis, Chandra Malladi for the PKC preparation and Professor Peter Rowe for comments on the manuscript. This work was supported by grants from the National Health and Medical Research Council of Australia (NHMRC to D.A.J. and R.R.R.), an Aza Research and Development grant and the Carcinogenesis Fellowship of the NSW Cancer Council.

REFERENCES

- Wagner, G. F., Vozzolo, B. L., Jaworski, E., Haddad, M., Kline, R. L., Olsen, H. S., Rosen, C. A., Davidson, M. B. and Renfro, J. L. (1997) Human stanniocalcin inhibits renal phosphate excretion in the rat. *J. Bone Miner. Res.* **12**, 165–171
- Madsen, K. L., Tavernini, M. M., Yachimec, C., Mendrick, D. L., Alfonso, P. J., Buerger, M., Olsen, H. S., Antonaccio, M. J., Thomson, A. B. R. and Fedorak, R. N. (1998) Stanniocalcin: a novel protein regulating calcium and phosphate transport across mammalian intestine. *Am. J. Physiol.* **274**, G96–G102
- Yoshiko, Y. and Maeda, N. (1998) *In situ* hybridization analysis of stanniocalcin mRNA expressing cells in the mouse kidney. *Mol. Cell. Endocrinol.* **141**, 37–40
- Varghese, R., Wong, C. K. C., Deol, H., Wagner, G. F. and Dimattia, G. E. (1998) Comparative analysis of mammalian stanniocalcin genes. *Endocrinology* **139**, 4714–4725
- Zhang, K., Westberg, J. A., Paetau, A., von Boguslawsky, K., Lindsberg, P., Erlander, M., Guo, H., Su, J., Olsen, H. S. and Andersson, L. C. (1998) High expression of stanniocalcin in differentiated brain neurons. *Am. J. Pathol.* **153**, 439–445
- Yoshiko, Y., Son, A., Maeda, S., Igarashi, A., Takano, S., Hu, J. and Maeda, N. (1999) Evidence for stanniocalcin gene expression in mammalian bone. *Endocrinology* **140**, 1869–1874
- Chang, A. C. M., Janosi, J., Hulsbeek, M., De Jong, D., Jeffrey, K. J., Noble, J. R. and Reddel, R. R. (1995) A novel human cDNA highly homologous to the fish hormone stanniocalcin. *Mol. Cell. Endocrinol.* **112**, 241–247
- Wendelaar Bonga, S. E. and Pang, P. K. T. (1991) Control of calcium regulating hormones in the vertebrates: parathyroid hormone, calcitonin, prolactin, and stanniocalcin. *Int. Rev. Cytol.* **128**, 139–213
- Chang, A. C. M. and Reddel, R. R. (1998) Identification of a second stanniocalcin cDNA in mouse and human: Stanniocalcin 2. *Mol. Cell. Endocrinol.* **141**, 95–99
- Dimattia, G. E., Varghese, R. and Wagner, G. F. (1998) Molecular cloning and characterization of stanniocalcin-related protein. *Mol. Cell. Endocrinol.* **146**, 137–140
- Moore, E. E., Kuestner, R. E., Conklin, D. C., Whitmore, T. E., Downey, W., Buddle, M. M., Adams, R. L., Bell, L. A., Thompson, D. L., Wolf, A. et al. (1999) Stanniocalcin 2: characterization of the protein and its localization to human pancreatic alpha cells. *Horm. Metab. Res.* **31**, 406–414
- Ishibashi, K., Miyamoto, K., Taketani, Y., Morita, K., Takeda, E., Sasaki, S. and Imai, I. (1998) Molecular cloning of a second human stanniocalcin homologue (STC2). *Biochem. Biophys. Res. Commun.* **250**, 252–258
- Massias, J. F., Hardouin, S., Vieau, D., Lenne, F. and Bertagna, X. (1994) Phosphorylated forms of adrenocorticotropin and corticotropin-like intermediary lobe peptide in human tumors. *Acta Endocrinol. (Copenhagen)* **131**, 341–346
- Saccuzzo, J. E., Krzesicki, R. F., Perini, F. and Ruddon, R. W. (1986) Phosphorylation of the secreted, free alpha subunit of human chorionic gonadotropin. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9493–9496
- Vilgrain, I. and Baird, A. (1991) Phosphorylation of basic fibroblast growth factor by a protein kinase associated with the outer surface of a target cell. *Mol. Endocrinol.* **5**, 1003–1012
- Kubler, D., Reinhardt, D., Reed, J., Pyerin, W. and Kinzel, V. (1992) Atrial natriuretic peptide is phosphorylated by intact cells through cAMP-dependent ecto-protein kinase. *Eur. J. Biochem.* **206**, 179–186
- Dautzenberg, F. M., Müller, D. and Richter, D. (1993) Dephosphorylation of phosphorylated atrial natriuretic peptide by protein phosphatase 2A. *Eur. J. Biochem.* **211**, 485–490
- 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
- Liberti, J. P. and Joshi, G. S. (1986) Synthesis and secretion of phosphorylated growth hormone by rat pituitary glands *in vitro*. *Biochem. Biophys. Res. Commun.* **137**, 806–812
- Kim, B. G. and Brooks, C. L. (1993) Isolation and characterization of phosphorylated bovine prolactin. *Biochem. J.* **296**, 41–47
- Maciejewski, P. M., Peterson, F. C., Anderson, P. J. and Brooks, C. L. (1995) Mutation of serine 90 to glutamic acid mimics phosphorylation of bovine prolactin. *J. Biol. Chem.* **270**, 27661–27665
- Wicks, J. R. and Brooks, C. L. (1995) Biological activity of phosphorylated and dephosphorylated bovine prolactin. *Mol. Cell. Endocrinol.* **112**, 223–229
- Robinson, P. J., Sontag, J. M., Liu, J. P., Fykse, E. M., Slaughter, C., McMahon, H. and Sudhof, T. C. (1993) Dynamin GTPase regulated by protein kinase C phosphorylation in nerve terminals. *Nature (London)* **365**, 163–166
- Robinson, P. J. and Dunkley, P. R. (1983) Depolarization-dependent protein phosphorylation in rat cortical synaptosomes: the effects of calcium, strontium and barium. *Neurosci. Lett.* **43**, 85–90
- Robinson, P. J., Liu, J. P., Chen, W. and Wenzel, T. (1993) Activation of protein kinase C *in vitro* and in intact cells or synaptosomes determined by acetic acid extraction of MARCKS. *Anal. Biochem.* **210**, 172–178
- Robinson, P. J. (1991) Dephosphin, a 96,000 Da substrate of protein kinase C in synaptosomal cytosol, is phosphorylated in intact synaptosomes. *FEBS Lett.* **282**, 388–392
- Dunkley, P. R., Baker, C. M. and Robinson, P. J. (1986) Depolarization-dependent protein phosphorylation in rat cortical synaptosomes: characterization of active protein kinases by phosphopeptide analysis of substrates. *J. Neurochem.* **46**, 1692–1703
- Gobom, J., Nordhoff, E., Mirgorodskaya, E., Ekman, R. and Roepstorff, P. (1999) Sample purification and preparation technique based on nano-scale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix-assisted laser desorption/ionization mass spectrometry. *J. Mass Spectrom.* **34**, 105–116
- Jensen, O. N., Larsen, M. R. and Roepstorff, P. (1998) Mass spectrometric identification and microcharacterization of proteins from electrophoretic gels: strategies and applications. *Proteins suppl.* **2**, 74–89
- Tuazon, P. T. and Traugh, J. A. (1991) Casein kinase I and II-multipotential serine protein kinases: structure, function, and regulation. *Adv. Second Messenger Phosphoprotein Res.* **23**, 123–164
- Walter, J., Schnolzer, M., Pyerin, W., Kinzel, V. and Kubler, D. (1996) Induced release of cell surface protein kinase yields CK1- and CK2-like enzymes in tandem. *J. Biol. Chem.* **271**, 111–119
- Kemp, B. E. and Pearson, R. B. (1990) Protein kinase recognition sequence motifs. *Trends. Biochem. Sci.* **15**, 342–346
- Gmeiner, B. M. (1987) Phorbol ester, retinoic acid and diacylglycerol decrease ecto- but increase total basal-protein kinase activity of human fibroblasts. *Biochem. Biophys. Res. Commun.* **143**, 600–608

- 34 Muramoto, K., Taniguchi, H., Kawahara, M., Kobayashi, K., Nonomura, Y. and Kuroda, Y. (1994) A substrate of ecto-protein kinase is microtubule-associated protein 1B in cortical cell cultures undergoing synaptogenesis. *Biochem. Biophys. Res. Commun.* **205**, 1467–1473
- 35 Hogan, M. V., Pawlowska, Z., Yang, H. A., Kornecki, E. and Ehrlich, Y. H. (1995) Surface phosphorylation by ecto-protein kinase C in brain neurons: a target for Alzheimer's β -amyloid peptides. *J. Neurochem.* **65**, 2022–2030
- 36 Geberhiwot, T. and Skoglund, G. (1995) Ectoprotein kinase activities on non-differentiated and differentiated U-937 cells. *Cell Signal.* **7**, 423–429
- 37 Babinska, A., Ehrlich, Y. H. and Kornecki, E. (1996) Activation of human platelets by protein kinase C antibody: role for surface phosphorylation in homeostasis. *Am. J. Physiol.* **271**, H2134–H2144
- 38 Kübler, D., Pyerin, W., Bill, O., Hotz, A., Sonka, J. and Kinzel, V. (1989) Evidence for ecto-protein kinase activity that phosphorylates kemptide in a cyclic AMP-dependent mode. *J. Biol. Chem.* **264**, 14549–14555
- 39 Chen, W., Wieraszko, A., Hogan, M. V., Yang, H. A., Kornecki, E. and Ehrlich, Y. H. (1996) Surface protein phosphorylation by ecto-protein kinase is required for the maintenance of hippocampal long-term potentiation. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8688–8693
- 40 Pyerin, W., Burow, E., Michaely, K., Kubler, D. and Kinzel, V. (1987) Catalytic and molecular properties of highly purified phosphatase/casein kinase type II from human epithelial cells in culture (HeLa) and relation to ecto protein kinase. *Biol. Chem. Hoppe Seyler* **368**, 215–227
- 41 Zhu, X., Luo, C., Ferrier, J. M. and Sodek, J. (1997) Evidence of ectokinase-mediated phosphorylation of osteopontin and bone sialoprotein by osteoblasts during bone formation in vitro. *Biochem. J.* **323**, 637–643
- 42 Walter, J., Kinzel, V. and Kubler, D. (1994) Evidence for CKI and CKII at the cell surface. *Cell. Mol. Biol. Res.* **40**, 473–480
- 43 Redegeld, F. A., Smith, P., Apasov, S. and Sitkovsky, M. V. (1997) Phosphorylation of T-lymphocyte plasma membrane-associated proteins by ectoprotein kinases: implications for a possible role for ectophosphorylation in T-cell effector functions. *Biochim. Biophys. Acta* **1328**, 151–165
- 44 Naik, U. P., Kornecki, E. and Ehrlich, Y. H. (1991) Phosphorylation and dephosphorylation of human platelet surface proteins by an ecto-protein kinase/phosphatase system. *Biochim. Biophys. Acta Mol. Cell. Res.* **1092**, 256–264
- 45 Hardwick, J. C., Ehrlich, Y. H. and Hendley, E. D. (1989) Extracellular ATP stimulates norepinephrine uptake in PC12 cells. *J. Neurochem.* **53**, 1512–1518
- 46 Sonka, J., Kubler, D. and Kinzel, V. (1989) Phosphorylation by cell surface protein kinase of bovine and human fibrinogen and fibrin. *Biochim. Biophys. Acta* **997**, 268–277
- 47 Kalafatis, M., Rand, M. D., Jenny, R. J., Ehrlich, Y. H. and Mann, K. G. (1993) Phosphorylation of factor Va and factor VIIIa by activated platelets. *Blood* **81**, 704–719
- 48 Feige, J. J., Bradley, J. D., Fryburg, K., Farris, J., Cousens, L. C., Barr, P. J. and Baird, A. (1989) Differential effects of heparin, fibronectin, and laminin on the phosphorylation of basic fibroblast growth factor by protein kinase C and the catalytic subunit of protein kinase A. *J. Cell. Biol.* **109**, 3105–3114
- 49 Forsberg, P. O. and Martin, S. C. (1990) Plasmin digestion of human fibrinogen previously phosphorylated by protein kinase C or dephosphorylated by alkaline phosphatase in vitro. *Thromb. Res.* **58**, 119–127
- 50 Heldin, P., Hessel, B., Humble, E., Blomback, B. and Engstrom, L. (1987) Effect of phosphorylation *in vitro* of human fibrinogen with protein kinase C on thrombin-induced gelation. *Thromb. Res.* **47**, 93–99

Received 12 November 1999/31 May 2000; accepted 20 June 2000