Myristoylated and Nonmyristoylated Pools of Sea Urchin Sperm Flagellar Creatine Kinase Exist Side-by-Side: Myristoylation Is Necessary for Efficient Lipid Association†

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ABSTRACT: In sperm of the sea urchin *Strongylocentrotus purpuratus*, a functional phosphocreatine shuttle, that requires the existence of mitochondrial and cytosolic creatine kinase (CK) isoforms in distinct locations, is essential for sperm motility. *S. purpuratus* sperm have an unusually large, 145 kDa CK isoform, present exclusively in the sperm tail (TCK), that is enriched in flagellum membrane preparations. Purified TCK contains two very similar proteins, designated TCKI and TCKII, of which only TCKII associates readily with liposomes and detergent micelles *in vitro*. Here we demonstrate by gas chromatography/mass spectrometry combined with selective ion monitoring that ions diagnostic for the presence of myristoyl-glycine in proteins are found in TCKII, but not TCKI. By contrast, TCKI, but not TCKII, served *in vitro* as a substrate for recombinant, polyhistidine-tagged *N*-myristoyltransferase and was myristoylated to high stoichiometries (0.58 ± 0.14 pmol of myristate/pmol of TCK), in the presence of myristoyl-CoA, on glycine in amide linkage. *In vitro* myristoylated TCKI associated with phosphatidylcholine (PC)/phosphatidylserine (PS) (75:25) liposomes and Triton X-100 detergent micelles in gel filtration assays and with PC/PS liposomes in a centrifugation assay in the same manner as did TCKII. In gel filtration experiments, TCKI required at least 25-fold higher PC/PS liposome concentrations than TCKII to obtain 50% association. A partition coefficient of 0.8×10^5 $M^{-1}$was determined for TCKII with PC/PS (75:25) liposomes in the centrifugation assay. Thus, myristoylated and nonmyristoylated forms of TCK exist side-by-side in the sea urchin flagellum, and myristoylation is essential for efficient liposome association of TCK.

Cytosolic sea urchin sperm tail creatine kinase (TCK)[1] is found exclusively in the flagellum, where its presence is critical for maintenance of a functional phosphocreatine shuttle and sperm motility (Tombes & Shapiro, 1985). Although a cytosolic protein, TCK is highly prominent in sperm tail membrane preparations (Quest & Shapiro, 1991a). Biochemical characterization of TCK purified from sea urchin sperm revealed the existence of two different pools of pure TCK, TCKI and TCKII, of which only TCKII associated readily with liposomes and detergent micelles *in vitro*. However, aside from this difference TCKI and TCKII appeared to be remarkably similar (Quest & Shapiro, 1991a). By expression of TCK in baby hamster kidney (BHK) cells, stably transfected with a cDNA encoding for TCK (Wothe et al., 1990) under the control of a metallothionine promotor, followed by myristate labeling, TCK was shown to be a myristoylated protein. Furthermore, evidence was provided demonstrating that myristoylated TCK from BHK cells behaved like purified TCKII in the liposome association assay, suggesting that myristoylation may be important for TCK association with lipids (Quest et al., 1992).

Myristoylation is generally considered a cotranslational, irreversible protein modification, where myristic acid is incorporated at an essential NH_2-terminal glycine residue by myristoyl-CoA:protein *N*-myristoyltransferase (NMT)-dependent acylation (Towler et al., 1988; Wilcox et al., 1987). However, in some cases, myristoylation may not necessarily occur cotranslationally (da Silva & Klein, 1990; McIlhinney

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[1] Abbreviations: Ac-TCK, iodoacetate-treated TCK; BHK cells, baby hamster kidney cells; BHT, butylated hydroxytoluene; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; cAdPK, cAMP-dependent protein kinase; DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; CK, creatine kinase; EGTA, ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FPLC, fast protein liquid chromatography; GC/MS, gas chromatography/mass spectrometry; G-protein, guanine nucleotide-binding regulatory protein; GST, glutathione ***S***-transferase; GST−PKC, recombinant fusion proteins between GST and protein kinase C; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; his-NMT, recombinant polyhistidine-tagged myristoyl-CoA:protein *N*-myristoyltransferase; HPLC, high-pressure liquid chromatography; (iv)myr-TCK, *in vitro* myristoylated TCK; MARCKS, myristoylated alanine-rich C-kinase substrate; Mi-CK, mitochondrial creatine kinase; MRP, MARCKS-related protein, also refered to in the literature as MacMARCKS or F52; NMT, myristoyl-CoA:protein *N*-myristoyltransferase; PC, phosphatidylcholine; PCr, phosphocreatine; PS, phosphatidylserine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCK, sea urchin sperm tail-specific creatine kinase; TFA, trifluoroacetic acid; TMS, trimethylsilane.

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& McGlone, 1990; Manenti et al., 1993; Vergeres et al., 1995). Furthermore, myristoylation on internal residues, such as lysine or cysteine, has also been reported (Hedo et al., 1987; Muszbek & Laposata, 1993; Pillai & Baltimore, 1987; Stevenson et al., 1992, 1993).

Many proteins, that are involved in intracellular signal transduction, like cAMP-dependent protein kinase (cAdPK), calcineurin B, G-proteins, and nonreceptor tyrosine kinases of the src family, are myristoylated, and the presence of the fatty acid is thought to play a role in their subcellular distribution and be important for their signalling potential (Towler et al., 1988). How precisely myristate contributes to intracellular protein distribution is still unclear. For $p60^{src}$, a myristate-dependent, cytoplasmic-face protein receptor may exist (Goddard et al., 1989; Resh, 1989; Resh & Ling, 1990), while for the myristoylated alanine-rich C-kinase substrate (MARCKS) membrane association results mainly from hydrophobic interactions between myristate and the phospholipid bilayer [see discussion in Blackshear (1993)]. Experiments *in vitro* with either MARCKS or the MARCKS-related protein (MRP) reveal clearly that the presence of myristate enhances the ability of both proteins to bind to phospholipids. However, myristoylation alone is not considered sufficient to permit stable membrane association of a protein (Kim et al., 1994; Vergeres et al., 1995). Additional protein elements mediating either ionic interactions (as for MARCKS, MRP, and $p60^{src}$) or hydrophobic interactions (as for $p21^{ras}$ and other src family members) are required for stable myristoyl−protein membrane interactions (Alland et al., 1994; Cadwallader et al., 1994; Koegl et al., 1994; Kim et al., 1994; Resh, 1994; Silverman & Resh, 1992; Vergeres et al., 1995).

In order to understand better at a molecular level the forces important for TCK association with sperm tail membranes (Quest & Shapiro, 1991a), we have characterized further the two purified TCK fractions, TCKI and TCKII. Of particular interest was the question whether the absence or presence of an NH_2-terminal myristate moiety may explain the differences in lipid association observed *in vitro* for TCKI and TCKII. We present evidence that purified TCKII is indeed myristoylated, while TCKI is not. Furthermore, upon myristoylation *in vitro* by recombinant NMT, TCKI associates readily with liposomes and detergent micelles in a manner identical to that of TCKII. These results suggest that TCKI is a nonmyristoylated sea urchin sperm tail creatine kinase, highly akin to TCKII, that may represent either a nonmyristoylated or a demyristoylated form of TCKII. The relevance of these observations for targetting of TCK to the sperm flagellum is discussed.

MATERIALS AND METHODS

Sperm were obtained from *Strongylocentrotus purpuratus*, and TCK fractions I and II prepared as described (Quest & Shapiro, 1991a). Dioleoylphosphatidylcholine and phosphatidylserine were from Avanti Polar Lipids Inc.; Triton X-100 from Pierce Chemical Co.; myristoyl-CoA from Sigma; and [^{3}H]myristoyl-CoA was prepared as described (McIlhinney et al., 1994); all other reagents were of the highest quality available unless stated otherwise.

Expression and Purification of Recombinant NMT. Recombinant, active NMT with an NH_2-terminal polyhistidine-tag (his-NMT) was expressed in *Escherichia coli* and subsequently affinity-purified as previously described (McIlhinney et al., 1994).

Table 1: Summary of *in Vitro* Myristoylation Experiments of TCKI and TCKII[a]

sample	stoichiometry (pmol of myristate/pmol of TCK)
TCKI, +NMT	0.58 ± 0.14 ($n = 4$)
AcTCKI, +NMT	0.54 ($n = 2$)
TCKII, +NMT	nd ($n = 3$)
AcTCKII, +NMT	nd ($n = 3$)

[a] TCK samples were myristoylated *in vitro* under conditions favoring high stoichiometry (see Materials and Methods). Stoichiometries (pmol of myristate/pmol of TCK) of TCKI and TCKII myristoylation in the presence of NMT are summarized. The number of experiments from which values were averaged is indicated (n). Unlike in Figure 3, no significant incorporation of labeled myristate was detectable here in the absence of NMT. TCKII samples were prepared in all experiments in parallel, but in most cases ($n = 3$) did not give detectable levels of myristate (nd) as evidenced by autoradiography and summarized in the table. In the other experiment, stoichiometries of myristate incorporation in the presence of his-NMT were 0.05 and 0.03 for nontreated and iodoacetamide-treated (Ac) TCKII, respectively. The error indicated for TCKI corresponds to the standard deviation of values obtained from independent experiments ($n = 4$).

In Vitro Myristoylation of Purified TCK. Purified TCK samples (about 10 μg) dialyzed against 50 mM Tris-HCl, pH 8.5, and 1 mM DTT overnight at 4 °C (final volume between 100 and 300 μL) were myristoylated *in vitro* using his-NMT (0.1 μg) in the presence of high-specific-activity [^{3}H]myristoyl-CoA. Depending on the aim of the experiment, the total concentration of myristoyl-CoA varied. In initial experiments (see Figure 3), myristoyl-CoA concentrations (0.1−0.2 μM) below the K_m of NMT (high specific activity) were employed to maximize sensitivity. As a result, myristoylation stoichiometries were lower in these experiments. Subsequently, to improve the stoichiometries of myristate incorporation, concentrations of myristoyl-CoA (10 μM) in excess of the K_m were employed (Table 1). In some control experiments to assess specificity, NMT was omitted. Alternatively, to prevent unspecific, nonenzymatic incorporation of myristate at cysteine residues, some TCK samples were treated with 5 mM iodoacetate (AcTCK) prior to *in vitro* myristoylation.

To determine labeling stoichiometries, TCK samples were separated after *in vitro* myristoylation from free myristoyl-CoA by SDS−PAGE, and the position of the labeled TCK was determined by fluorography using diphenyloxazole as the fluor (Laskey & Mills, 1975). The bands were excised and rehydrated with distilled water for 30 min at room temperature, and the diphenyloxazole was removed by two 30 min treatments with dimethyl sulfoxide (5 mL). The cleansed gel was transferred to a screw-capped vial, incubated with 0.5 mL of Soluene-100 (Packard Instruments Co.) for 24 h at 37 °C. Released radioactivity was determined by scintillation counting using 5 mL of Liquiscint (National Diagnostics).

Using this method, stoichiometries of 0.5−0.7 pmol of myristate/pmol of protein were obtained for both TCK and MRP. The latter protein shows a gel shift following myristoylation, and visual inspection of Coomassie Blue stained gels of *in vitro* myristoylated MRP indicated that the proportion of myristoylated MRP was significantly higher than when assessed by incorporation of radioactivity. Thus, the determination of myristate incorporation by radioactivity,

for both TCK and MRP, should be regarded as a minimum estimate of the actual incorporation of the fatty acid. In addition, stoichiometry estimates are lowered for TCKI by the presence of 20% TCKII, a nonmyristoylatable TCK species, in TCKI preparations (see Discussion).

HPLC Analysis of [^{3}H]Myristate-Labeled TCK after Pronase Treatment. To determine the amino acid to which myristate was attached *in vitro*, myristoylated TCKI samples were exhaustively digested with Pronase (McIlhinney & McGlone, 1990), and the released radioactivity was extracted into chloroform/methanol (1:1 v/v). Released radioactivity was analyzed by reversed phase chromatography using C18 reversed phase silica matrix (VYDAC 15−20 μm) in a hand-packed 0.5 × 5.0 cm column (Pharmacia HR 5/5) run from 0−90% acetonitrile in 0.1% TFA at 1 mL/min (McIlhinney, 1992). Fractions of 1 mL were collected and counted. The positions of myristoylglycine [prepared as described in McIlhinney (1992)] and free myristic acid (Sigma) were determined by monitoring the absorbance of standards at 214 nm.

GC/MS Analysis of Myristoylglycine Derived from Purified Proteins: Comparison of TCKI and TCKII. All methods mentioned briefly in this section have been described in greater detail elsewhere (McIlhinney & Harvey, 1995).

Mass spectra were obtained with a Micromass Autospec-Q-mass spectrometer interfaced to a Hewlett-Pakard 5890 Series II gas chromatograph containing a 15 M OV-1 bonded-phase fused silica capillary column. The mass spectrometer was operated under the following conditions: accelerating voltage, 8 kV; electron energy, 70 eV; trap current, 200 mA; ion source temperature, 250 °C; scan speed for full spectra, 1 s decade^{-1}. The gas chromatographic conditions were as follows: injector temperature, 300 °C; oven temperature programmed from 160 to 250 °C at 10 °C min^{-1}; carrier gas (helium flow), 1 mL min^{-1}.

For GC/MS analysis, trimethylsilyl (TMS) esters of myristoylglycine were prepared. Dry acid hydrolysate (10 μmol) was dissolved in pyridine (1 mL). A mixture of this solution (2 μL) with 18 μL of *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was heated to 60 °C for 10 min. Aliquots of 1 μL were injected either directly into the gas chromatograph or after serial dilution by 1:10 with BSTFA prior to injection.

The mass spectrum of the TMS derivative of myristoylglycine is highly characteristic. The following ions at *m/z* 145, 158, 172, and 189 are abundant and present in the spectra of all saturated acylglycines. In addition, the $[M-CH_3]^+$ ion at *m/z* 342 is diagnostic of myristoylglycine. Using these ions, a selective ion monitoring method was employed for the detection of myristoylglycine. Each ion was integrated for 80 ms. Following this protocol, 100 fmol of myristoylglycine can be detected with a 3:1 signal-to-noise ratio.

To demonstrate the applicability of the method to a large protein, purified TCK samples were compared with a standard myristoylglycine preparation (McIlhinney & Harvey, 1995) and three synthetic peptides myristoylated *in vitro*, derived from p60^{src} , MARCKS, and the cAdPK catalytic subunit. The terminal acylglycine was cleaved from the peptides or proteins in 50 mM HCl at 100 °C for 6 h in sealed Reactivials (Pierce Chemical Co.) which were then cooled and the contents freeze-dried. The dried hydrolysate was redissolved in distilled water and freeze-dried again. These conditions result in partial hydrolysis of the peptides with maintenance of the amide bond of myristoylglycine (Goddard & Felsted, 1988). Either buffer alone or IgG (Calbiochem) processed in the same manner served as negative controls for the following steps. Routinely, about 200 fmol of protein was analyzed as described in each experiment.

Liposome Association Assessed by Centrifugation Assay. These experiments were done as previously described (Quest et al., 1994) with some modifications. Briefly, phosphatidylcholine (PC)/phosphatidylserine (PS) (75:25) mixtures were prepared by drying down ethanolic aliquots in a stream of nitrogen and resuspending by sonication (see above) in aqueous buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 5 μg/mL BHT, and 0.1 mM DTPA) to yield lipid stock solutions with up to 1 mg/mL final lipid concentration. TCK samples (0.5 μg) in the presence of GST carrier (2.5 μg) were incubated with liposomes at the concentrations indicated in a final volume of 180 μL for 20−30 min at room temperature and subsequently cooled on ice for 15 min. Lipid-associated protein was collected in the pellet obtained after centrifugation for 15 min at 4 °C in a Beckman Airfuge at 100000*g*. After centrifugation, samples were immediately placed on ice, and the supernatant was removed. The liposome pellet was resuspended in 100 μL of buffer and 5 μL of 10% Triton X-100. Both supernatant and pellet fractions were then chlorofom/methanol-precipitated in the presence of GST carrier protein and analyzed by SDS−PAGE.

To characterize lipid interactions of TCKII, a partition coefficient was calculated as previously described by McLaughlin and co-workers for MARCKS (Kim et al., 1994).

Gel Filtration Analysis of TCKI and -II. TCK samples were characterized by gel filtration on an FPLC Superose 12 HR10/30 column (Pharmacia LKB Biotechnology Inc.) in buffer G (11% ethyleneglycol, 111 mM KCl, 5.5 mM Tris, 22 mM HEPES, 1.1 mM EGTA, and 1.1 mM DTT, pH adjusted to 8 with KOH) at a flow rate of 0.25−0.33 mL/min. In all experiments, 200 μL sample volumes were injected onto the column, and fractions of 0.5 mL were collected. TCKI, TCKII, and *in vitro* myristoylated TCKI [(iv)myr-TCKI] samples were analyzed either in buffer G alone, or with 1.4% Triton X-100, or in the presence of PC/PS (75:25) liposomes at concentrations up to 10 mg/mL.

Liposome and Detergent Micelle Association Assessed by Gel Filtration Assay. A gel filtration assay characterizing the distinct lipophilic properties of purified TCKI and TCKII described in detail elsewhere (Quest & Shapiro, 1991a; Quest et al., 1992) was used with some modifications. Briefly, PC/PS (75:25) stock solutions at 10.8 mg/mL were prepared by drying down ethanolic aliquots in a stream of nitrogen and resuspending in modified buffer G with 5 μg/mL butylated hydroxytoluene (BHT) and 0.1 mM diethylenetriaminepentaacetic acid (DTPA) by sonication (3 × 15 s) in a bath sonicator (Branson Cleaning Equipment Co.) and cooling intermittently on ice. Samples (4 μg) of purified TCKs, either alone or in the presence of (iv)myr-TCKI, were diluted 1:10 either in buffer G alone or in buffer G with PC/PS liposomes at various concentrations. Samples were incubated for 60 min at room temperature and subsequently cooled to 4 °C on ice for 30 min prior to separation. Samples of 200 μL were analyzed by gel filtration through an FPLC

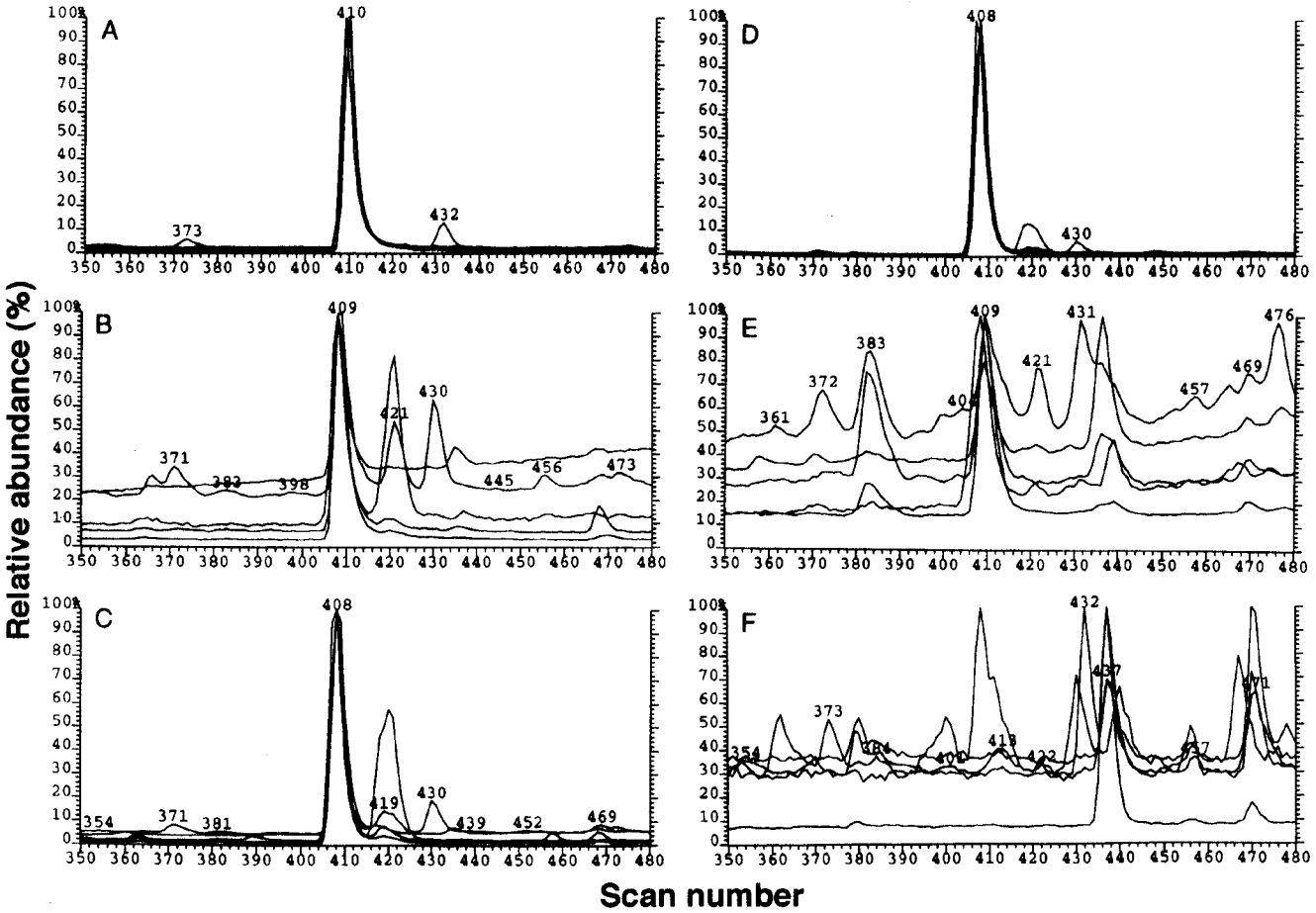

FIGURE 1: Comparison by GC/MS of TCKII with peptides from proteins known to be myristoylated *in vivo*: selected ion monitoring for extracted myristoylglycine. Myristoylglycine from a standard solution (panel A) and derived from several synthetic peptides of myristoylated proteins ($p60^{src}$, MARCKS, and cAdPK catalytic subunit; panels B, C, and D, respectively) and purified TCKII samples were prepared and analyzed as described above. The ions monitored were *m/z* 145, 158, 172, 189, and 281 (lock mass, not included in figure) and 342 ($[M - CH_3]^+$), diagnostic of myristoylglycine. The presence of myristoylglycine is shown by coincident maxima in the abundance of these ions in the region of scan 408−410. This peak is also seen in samples derived from TCKII (panel E), but is absent in both a buffer blank (not shown) and an IgG control (panel F). Ions have been normalized to the most abundant peak in each mass trace. [Panels A and B are reproduced from McIlhinney and Harvey (1995) with permission from J. Wiley and Sons Ltd.]

Superose 12 HR 10/30 column (Pharmacia LKB Biotechnology Inc.) at 0.25−0.33 mL/min. Fractions (0.5 mL) were collected, precipitated in chloroform/methanol in the presence of 4 μg of glutathione *S*-transferase (GST) as a low molecular weight carrier protein, and analyzed by SDS−PAGE. To facilitate the evaluation of experiments, fractions 13−16 and 19−22 were pooled as liposome-associated or free TCK, respectively.

In the detergent micelle association experiments, TCK samples were incubated overnight at 4 °C in buffer G at pH 8 containing 0.7% Triton X-100 before initiating separation by gel filtration. Otherwise, samples were processed in the same manner as described above.

SDS−PAGE Analysis and Fluorography. Samples precipitated in chloroform/methanol and resuspended in sample buffer were analyzed by SDS−PAGE on 6−10% mini gels (Bio-Rad). After separation, gels were stained with Coomassie Blue. Tritium-labeled radioactive protein bands were visualized by treatment in 1 M sodium salicylate, pH 7, for 30 min and subsequent fluorography. Film (XAR-5, Kodak) exposure times varied from 10 days to 8 weeks. Both Coomassie Blue-stained gels and X-ray films were quantitated by scanning densitometry using AppleScan and the NIH Image 1.40 program.

RESULTS

GC/MS Analysis of TCKs. To provide definitive evidence that TCKII purified from sea urchin sperm tails is indeed myristoylated, a GC/MS method, previously employed to identify the presence of acylglycine in acylated synthetic peptides (McIlhinney & Harvey, 1995), was used. The mass spectrum of the TMS derivative of myristoylglycine is highly characteristic, with ions at *m/z* 145, 158, 189, 281, and 342 that are diagnostic. The presence of myristoylglycine in a standard preparation is evidenced by coincident maxima in the abundance of these ions in the region of scan 410 upon selective ion monitoring (see Figure 1, panel A; McIlhinney & Harvey, 1995). This peak is clearly visible also in the scans from *in vitro* myristoylated synthetic peptides with sequences corresponding to the NH_2-terminus of $p60^{src}$, MARCKS, and the cAdPK catalytic subunit (Figure 1, panels B, C, and D, respectively). Likewise, this peak is very prominent in scans of material derived from TCKII (Figure 1, panel E), while being absent in scans from control experiments employing either IgG (Figure 1, panel F) or just buffer alone (data not shown).

Since TCKI is a TCK fraction that did not associate with liposomes in initial experiments (Quest & Shapiro, 1991a), it was intriguing to speculate that TCKI might represent a

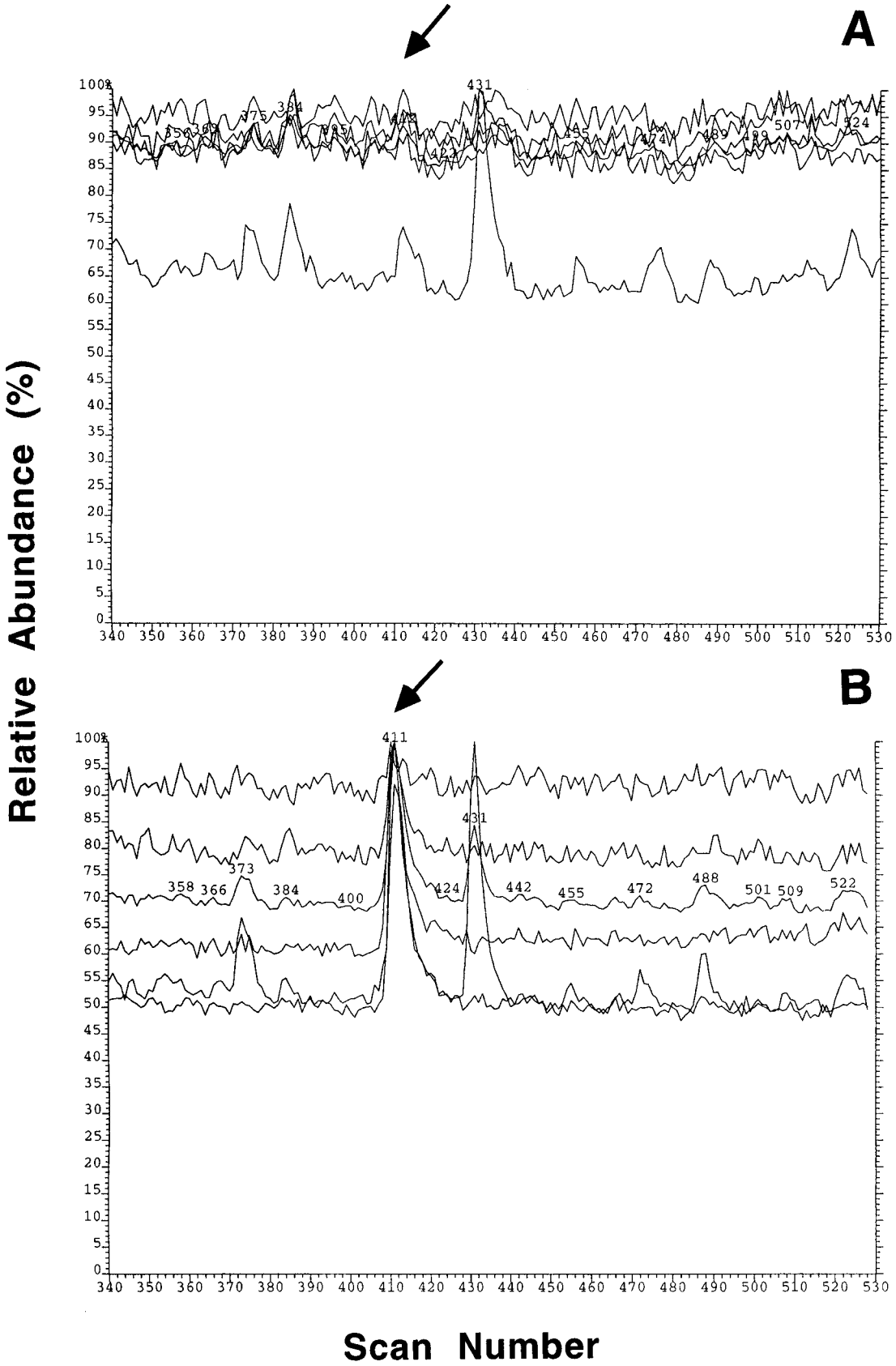

FIGURE 2: Comparison by GC/MS of TCKI and TCKII: selected ion monitoring for extracted myristoylglycine. TCKI and TCKII were processed for selective ion monitoring as described (Figure 1). Chromatograms are shown for TCKI and TCKII. The chromatograms on the right show an expansion of the region containing myristoylglycine. The peak in the region of scan 411, diagnostic of myristoylglycine in the original sample, is clearly visible for the TCKII-derived sample while being absent for TCKI. Ions in the TCKII chromatogram are, from top to bottom, *m/z* 281 (lock mass), 172, 342 ($[M - CH3]^+$), 189, 158, and 145. The several peaks appearing on the *m/z* 145 trace reflect the additional common occurrence of this ion in the spectra of TMS derivatives of compounds containing two or more TMS groups.

nonmyristoylated TCK variant. Indeed, a comparison of TCKI and TCKII by GC/MS analysis (Figure 2, see arrows) revealed that the peak diagnostic of myristoylglycine at 411 in selected ion monitoring scans was visible for TCKII

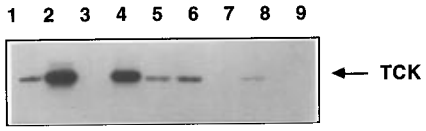

FIGURE 3: *In vitro* myristoylation of purified TCK by recombinant polyhistidine-tagged NMT: analysis by SDS−PAGE. TCK samples (1.5 μg of protein) were myristoylated *in vitro* under conditions favoring high specific labeling (see Materials and Methods) and subsequently analyzed by SDS−PAGE. The region of the Coomassie Blue stained gel where TCK samples migrated (M_r 145 000) is shown after autoradiography (16 h exposure): TCKI (lanes 1, 2) or TCKII (lanes 5, 6) myristoylated with [^{3}H]myristoyl-CoA either in the absence (lanes 1, 5) or in the presence (lanes 2, 6) of NMT; TCKI (lanes 3, 4) or TCKII (lanes 7, 8) pretreated with iodoacetate and then myristoylated with [^{3}H]myristoyl-CoA either in the absence (lanes 3, 7) or in the presence (lanes 4, 8) of NMT. Average (2 experiments) labeling stoichiometries (pmol of myristate/pmol of TCK expressed in %) under the conditions indicated are as follows: (1) 0.37; (2) 8.6; (3) 0.79; (4) 6.9; (5) 0.2; (6) 1.1; (7) 0.13; (8) 0.27. Equal amounts of TCK protein were present in lanes 1−8. The background in the M_r 145 000 range due to NMT alone in the presence of [^{3}H]myristoyl-CoA is illustrated in lane 9.

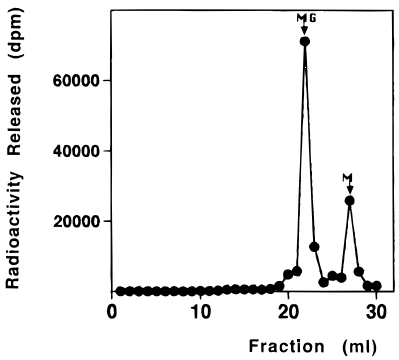

FIGURE 4: Myristic acid incorporated into TCKI is amide-linked to a glycine residue. After *in vitro* myristoylation, TCKI was digested with Pronase, and the released label was extracted in chloroform/methanol and analyzed by reversed phase chromatography as described. The radioactivities (dpm) measured in fractions (mL) from the column are shown. Arrowheads indicate the elution positions of myristoylglycine (MG) and myristic acid (M). Elution volumes were 22.4 and 28 mL, respectively.

(Figure 2, panel B) but not TCKI (Figure 2, panel A) preparations.

In Vitro Myristoylation of TCK. A trivial explanation for the lack of myristoylglycine in TCKI samples, despite the presence of multiple protease inhibitors throughout the purification, may have been that TCKI was derived from TCKII by proteolytic cleavage at the NH_2-terminus. An NH_2-terminal glycine residue is absolutely essential to ensure NMT-dependent myristoylation *in vivo* (Jones et al., 1990; Mumby et al., 1990). Thus, it was anticipated that TCKI should not be myristoylated *in vitro* by recombinant his-NMT if indeed TCKI were derived from TCKII by proteolytic cleavage at the NH_2-terminus. By contrast, a demyristoylated or nonmyristoylated form of TCKII could potentially serve as a substrate for myristoylation *in vitro*.

Previous experiments had shown that his-NMT would readily myristoylate synthetic peptides corresponding to sequences found at the NH_2-terminus of proteins myristoylated *in vivo*, such as the cAdPK catalytic subunit, p60^{src}, and MARCKS. Unlike these peptides, the actual proteins were generally far poorer substrates for his-NMT (see Discussion). Assuming TCKI would be poorly myristoylated, if at all, myristoylation conditions *in vitro* were initially chosen to optimize incorporation of labeled myristate into TCK, namely, high specific activity [^{3}H]myristoyl-CoA at total concentrations below the K_m of his-NMT for myristoyl-CoA. Results representative of at least three experiments under these conditions (Figure 3) revealed that myristate was incorporated at least 10-fold more efficiently into TCKI than TCKII in the presence of his-NMT under the same conditions (Figure 3, compare lanes 2 and 6, or 4 and 8). In the absence of his-NMT, comparable low levels of [^{3}H]myristate were incorporated into both TCKI and TCKII (Figure 3, lanes 1 and 5, respectively).

A concern in these experiments was that myristate could be covalently linked to cysteine residues, since posttranslational protein-*S*-fatty acid acylation is not specific for palmitate, but also occurs with myristate (Muszbek & Laposata, 1993). Several cysteines are present throughout the TCK sequence, one of which resides within the NH_2-terminal consensus for myristoylation, GCAASSQQ (Wothe et al., 1990; Quest et al., 1992). To prevent the reaction of myristoyl-CoA with sulfhydryl groups, TCKs were also pretreated with iodoacetate (AcTCK) in some experiments. This treatment essentially abolished [^{3}H]myristate incorporation into TCKs in the absence of his-NMT (Figure 3, lanes 3 and 7), and most label incorporated into TCKII (Figure 3, lane 8) but not TCKI (Figure 3, lane 4) in the presence of his-NMT. In the absence of TCK protein in a myristoylation reaction including his-NMT, no incorporation was visible after radiography in the region where TCK migrated (M_r 145 000) (Figure 3, lane 9).

To identify, unequivocally, whether the labeling detected in the above reaction was indeed due to covalent amide linkage between [^{3}H]myristate and the putative NH_2-terminal glycine residue of TCKI, (iv)myrTCKI was digested with Pronase, and radioactivity released was analyzed by reversed phase chromatography after chloroform/methanol extraction (Figure 4). Two peaks of radioactivity were eluted in an acetonitrile gradient from the column with retention times correlating precisely with those of the standards myristoylglycine (22.4 mL) and myristic acid (28 mL). Thus, a free glycine residue is present at the NH_2-terminus of TCKI in a favorable context for *in vitro* myristoylation.

Compared with results obtained for *in vitro* myristoylation of the cAdPK catalytic subunit (stoichiometries ≤1%), TCKI was remarkably efficiently myristoylated (stoichiometries ≤10%), despite low, nonoptimal myristoyl-CoA concentrations (0.1−0.2 μM). To assess whether myristoylation stoichiometries could be improved, additional experiments were done in the presence of myristoyl-CoA at 10 μM concentrations above the K_m (7.6 μM) of his-NMT (McIlhinney et al., 1994). Results from such experiments are summarized in Table 1. Under these conditions, no incorporation of [^{3}H]myristate was observed in the absence of his-NMT (data not shown). However, as before (Figure 3), TCKI was 10-fold more efficiently myristoylated than TCKII, and levels of myristoylation achieved for TCKI in the presence of his-NMT(≤60%) were remarkably high, being comparable to those attained with the MARCKS protein (Schlieff et al., 1996).

Liposome Association of TCK: Centrifugation Assay. The great similarity between TCKI and TCKII (Quest & Shapiro, 1991a) other than the difference in myristoylation suggested that TCKI might represent a nonmyristoylated form of

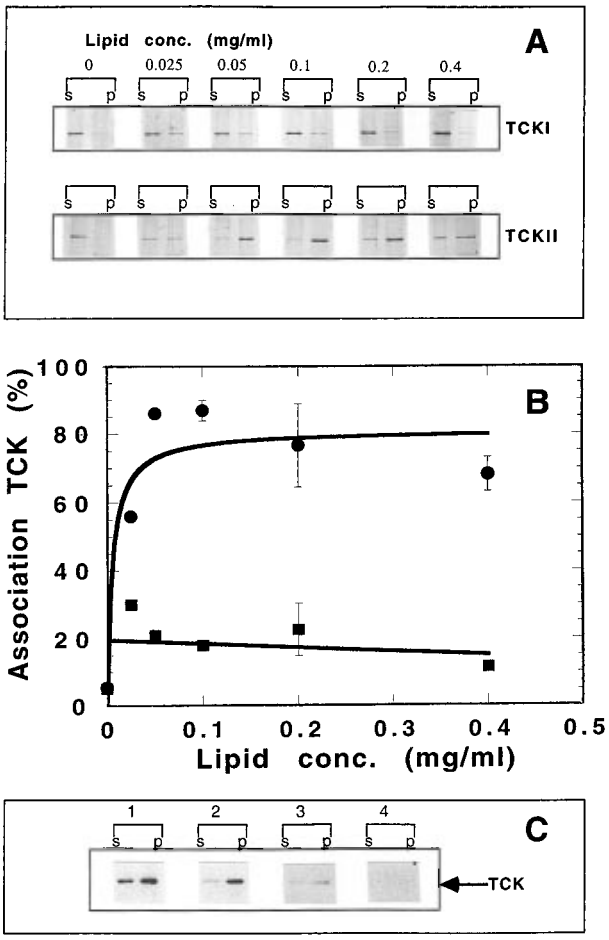

FIGURE 5: Association of TCKI and TCKII with PC/PS (75:25) liposomes: centrifugation assay. TCK samples (0.5 μg) were incubated with various concentrations of PC/PS (75:25) liposomes and bound (p) separated from free (s) TCK by centrifugation. Samples of TCK were subsequently analyzed by SDS−PAGE. The region of the gel where TCK migrates (M_r 145 000) is shown in representative experiments for TCKI (■) and TCKII (●). Averages from several such experiments for TCKI and TCKII evaluated by scanning densitometry are shown in panel B. Where three or more values were available, the error is indicated as the standard deviation of the values averaged. Average associations calculated for TCKI and TCKII were 23 ± 6% and 80 ± 6%, respectively. TCK recovery in the pellet in the absence of lipid was less than 10%. *In vitro* myristoylated TCKI (1, 2) and TCKII (3, 4) samples (0.5 μg) analyzed in the same manner are shown after autoradiography for 5 weeks in panel C. Liposome-bound (p) or free (s) TCKs were determined in the presence of 0.4 mg/mL total lipid concentration, whereby both nonpretreated (1, 3) and iodoacetate-pretreated (2, 4) TCK fractions were characterized. Note that *in vitro* myristoylated TCKI associates readily with liposomes. Average association is 80 ± 10% (n = 4).

TCKII. One prediction, if indeed this were the case, was that *in vitro* myristoylated TCKI should associate with lipids like TCKII. To test this hypothesis, a liposome association assay permitting characterization of the initially small quantities of labeled TCK available was used. This assay had previously been employed to characterize lipid interactions of GST−PKC fusion proteins (Quest et al., 1994). TCK was incubated with different concentrations of PC/PS (75:25) liposomes (Figure 5), and protein that associated with liposomes was recovered in the lipid pellet after centrifugation. Both lipid-bound (p) and free (s) TCKs were subsequently analyzed by SDS−PAGE. An experiment comparing TCKI and TCKII is shown after Coomassie Blue staining

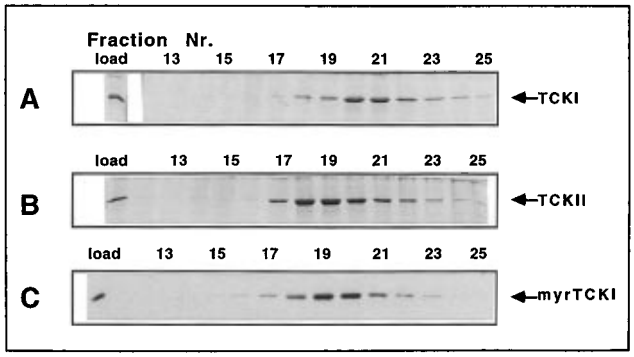

FIGURE 6: Association of TCKI and *in vitro* myristoylated TCKI and TCKII with Triton X-100 detergent micelles: gel filtration assay. TCK samples (4 μg) were analyzed by gel filtration in the presence of Triton X-100 detergent micelles. Fractions (0.5 mL) from the column were chloroform/methanol-precipitated and separated by SDS−PAGE. The region of the gel where TCK migrated (M_r 145 000) is shown. Material prior to gel filtration (load) and column fractions are shown either after Coomassie Blue staining (panels A, B) or after autoradiography (panel C). The shift in molecular weight due to association with detergent micelles (100 kDa) is less apparent than in the presence of liposomes. Thus, the peak of micelle-associated TCKII appears in fractions 18/19 (panel B), displaced by two fractions from the peak for TCKI (panel A) which essentially coelutes with free TCKII (not shown). Note the presence of a faintly visible protein band migrating slightly above TCKI in panel A, that displays a distribution identical to that of TCKII (panel B) and *in vitro* myristoylated TCKI (panel C). The autoradiography shown in panel C was exposed for 6 weeks.

of the gel (Figure 5A). For TCKII, association of the protein with liposomes (p) is evident, while this is not the case for TCKI, where most of the protein was recovered in the unbound (s) fraction. Results from several such experiments that were numerically evaluated by scanning densitometry are also summarized (Figure 5B). On an average, levels of association detected for TCKII were 80 ± 6% and 23 ± 6% for TCKI. These values correlate well with those reported previously using a gel filtration assay (Quest & Shapiro, 1991a). Association seen with TCKI samples is attributed to a contamination of TCKI preparations with TCKII (Quest & Shapiro, 1991a; see also Figure 6A and Discussion).

Subsequently, (iv)myr-TCKI and -TCKII were analyzed in the same manner. An autoradiogram from a representative experiment using lipid concentrations of 0.4 mg/mL is shown (Figure 5C). Clearly, both (iv)myr-TCKI (lanes 1s/p) and (iv)myr-TCKII (lanes 3s/p) behaved very much alike, essentially mimicking the properties of TCKII (Figure 5A,B). For (iv)myr-TCKI, association was estimated to be 80 ± 10% (n = 4) as determined by scanning densitometry. Pretreatment of TCKI with iodoacetate (lanes 2s/p), which was used in initial experiments to prevent covalent linkage of myristate in particular to sulfhydryl groups of cysteines, did not reduce the degree of association seen for (iv)myr-TCKI. As expected based on results shown in Figure 3, (iv)-myr-TCKII was no longer detectable by autoradiography after iodoacetamide treatment (Figure 5C, lanes 4s/p).

Liposome and Detergent Micelle Association: Gel Filtration Assay. A major disadvantage of the above liposome assay is its high sensitivity to different buffer components that reduce liposome recovery, which is routinely on the order of 80% (Quest et al., 1994). As summarized in Table 1, stoichiometries of TCKI myristoylation were greatly improved by increasing the myristoyl-CoA concentration to 10 μM. However, such TCK samples associated only poorly

with liposomes in the centrifugation assay. Reduction, in this case, correlated with a decline in liposome recovery (data not shown), presumably due to the 100-fold elevated presence of myristoyl-CoA.

To avoid such problems, TCK samples were analyzed in subsequent experiments by gel filtration after incubation with PC/PS liposomes (see Materials and Methods). As expected based on previous results (Quest & Shapiro, 1991a), TCKII associated readily with PC/PS (75:25) liposomes (85−90%), while this was not the case for TCKI (17−18%). Only at the highest lipid concentration tested (10 mg/mL) was TCKI association increased to 48% (data not shown). By comparison, (iv)myrTCKI behaved like TCKII (93% association) when tested at two different lipid concentrations (2.7 and 9.7 mg/mL), corroborating the results obtained using the centrifugation assay (data not shown).

Additionally, the ability of TCKI, TCKII, and (iv)myr-TCKI to associate with Triton X-100 detergent micelles was compared (Figure 6). Due to the smaller size of Triton X-100 micelles (about 100 kDa), the shift in apparent molecular weight upon association is less obvious [see also Quest and Shapiro (1991a)]. An experiment is shown comparing the elution profile of TCKII (Figure 6A) and TCKI (Figure 6B) in the presence of micelles. It is important to note that the bulk of protein present in TCKI preparations behaves like TCKII in the absence of liposomes or micelles (data not shown) eluting from the column with a peak of protein in fractions 20/21/22. By contrast, the TCKII peak is displaced to fractions 18/19/20, reflecting an increase in apparent molecular weight upon micelle association. Gel filtration of (iv)myr-TCKI in the presence of Triton X-100 micelles revealed that *in vitro* myristoylated TCKI behaved like TCKII (Figure 6C).

DISCUSSION

S. purpuratus sperm have an unusually large, 145 kDa creatine kinase (CK) isoform, present exclusively in the sperm tail (TCK). Upon purification of TCK, three pools of activity, termed TCKI, TCKII, and TCKIII, were characterized in the final purification step on an FPLC MonoQ column. Subsequent studies have focused on the two pure enzyme fractions, TCKI and TCKII, that differ in their ability to associate with liposomes or detergent micelles (Quest & Shapiro, 1991a).

The TCK protein sequence, delineated from a full-length cDNA, revealed the presence of 3 internal repeats highly homologous to mammalian CK sequences, that are interspersed and flanked at the COOH-terminal end by short non-CK-like elements of 19, 20, and 13 amino acids, respectively (Wothe et al., 1990). The longest non-CK-like sequence of 60 amino acids found at the NH_2-terminus contains a consensus for myristoylation and indeed supports myristoylation *in situ* upon heterologous expression of TCK in either BHK cells or NIH-3T3 fibroblasts. Furthermore, myristoylated TCK from BHK cells associated with PC liposomes in a manner akin to TCKII, suggesting that myristoylation may be important for the lipid-association properties of TCKII (Quest et al., 1992). The detection by GC/MS of myristoylglycine in TCKII shown here (Figures 1 and 2) demonstrated unequivocally that this TCK fraction, purified from sea urchin sperm tails, was myristoylated.

Myristoylation is considered a cotranslational, irreversible protein modification (Towler et al., 1988; Wilcox et al., 1987), although some exceptions to this rule exist. For instance, a nonmyristoylated pool of MARCKS has been identified in both rat and bovine brains (McIlhinney & McGlone, 1990; Manenti et al., 1993). Also, myristoylation of p68 from *Dictyostelium discoideum* is a highly transient, posttranslational event (da Silva & Klein, 1990). Therefore, we tested whether TCKI might represent a nonmyristoylated TCK variant, a suspicion that was then confirmed by GC/MS analysis (Figure 2). Furthermore, TCKI was efficiently myristoylated in the presence of his-NMT, while TCKII was not (Figure 3, Table 1), eliminating the possibility that TCKI was derived from TCKII by proteolytic cleavage at the NH_2-terminus. In all cases, NMT-dependent TCKII myristoylation never exceeded 10% of the values measured with TCKI (Figure 3) and dropped to essentially nondetectable levels when conditions for myristoylation were optimal (Table 1).

Myristoylation on internal cysteine and lysine residues has also been reported (Hedo et al., 1987; Muszbek & Laposata, 1993; Pillai & Baltimore, 1987; Stevenson et al., 1992, 1993). To eliminate possible myristoylation on cysteine, TCK samples pretreated with iodoacetate were also tested as substrates for his-NMT. For both TCKI and TCKII, acetylation essentially abolished myristate incorporation in the absence of his-NMT, indicating that nonenzymatic covalent binding of myristate to TCK did occur (Figure 3). Myristoylation of TCKI in the presence of his-NMT was reduced by 20% upon the pretreatment with iodoacetate when low concentrations of myristoyl-CoA were used (Figure 3), but only by 10% when high myristoyl-CoA concentrations were present during the myristoylation reaction (Table 1). Thus, while a small amount of myristate may be attached to cysteine *in vitro*, this is not the case for the majority incorporated into TCKI. In addition, HPLC analysis of radiolabeled material released by Pronase treatment from *in vitro* myristoylated TCKI showed that myristate was attached to an NH_2-terminal glycine, thereby also ruling out the possible linkage of myristate to lysine residues (Figure 4).

The efficiency with which his-NMT posttranslationally incorporates myristate into TCKI is remarkable. Stoichiometries of myristoylation of 1% or less are more commonly observed using substrates such as the catalytic subunit of cAdPK (McIlhinney et al., 1994), which is stoichiometrically modified *in vivo* (Herberg et al., 1993). However, it is interesting to note that for MARCKS and MRP the stoichiometry of *in vitro* myristoylation is 50−70% (Schlieff et al., 1996), similar to values shown for TCKI (Table 1). Also, evidence for the presence of a pool of nonmyristoylated MARCKS protein *in vivo* has been provided (Manenti et al., 1993). These observations could suggest that MARCKS, MRP, and TCK belong to a group of proteins, that are either demyristoylated following synthesis, or whose myristoylation is regulated so as to produce a proportion of nonmyristoylated protein.

To demonstrate that myristoylation was required for TCK interaction with lipids, several preparations of (iv)myr-TCKI were compared in this repect with TCKII. Two different assays, both utilizing PC/PS (75:25) liposomes, were employed. In the gel filtration assay (data not shown), results obtained previously with liposomes of crude PC preparations from soybean lipids (Quest & Shapiro, 1991a) were corroborated. TCKII readily associated with PC/PS liposomes, while for TCKI association did not exceed 20% except at the highest lipid concentration (10 mg/mL) tested.

Experiments employing mixed detergent micelles have shown that the presence of negative charges (cholate) on the nonionic Triton X-100 micelle surface enhance considerably the rate of TCKII association, indicating that electrostatic interactions, while not being essential, could facilitate the association detected (Quest & Shapiro, 1991a). Since liposome association was previously quantitated using crude PC preparations, elevated association of TCKI at the highest concentration of PC/PS liposomes tested here may have resulted from additional electrostatic interactions due to the presence of PS. This interpretation is consistent with the observation that TCKII also binds to PC/PS liposomes in the gel filtration experiments discussed here roughly 25-fold more efficiently than previously reported (apparent K_d 4×10^3 M^{-1} vs 1.6×10^2 M^{-1}, respectively). This difference is similar to what has been reported for MARCKS protein association with electrically negative versus neutral lipids (Kim et al., 1994).

The apparent dissociation constant determined in the centrifugation assay (Figure 5) was significantly higher (0.8×10^5 M^{-1}). Discrepancies between values obtained in these two assays can be attributed to differences in the speed of separation of bound and free TCK, as well as sample dilution that occurs during gel filtration. The value obtained here for TCKII in the centrifugation assay is similar to the partition coefficient of MARCKS (4.3×10^5 M^{-1}) with vesicles that closely mimic the plasma membrane inner leaflet lipid composition (Kim et al., 1994).

In both the centrifugation assay (Figure 5) and the gel filtration (data not shown) assay (iv)myr-TCKI associated readily with liposomes with association values of $80 \pm 10\%$ ($n = 4$) and 93%, respectively. Additionally, TCK association with Triton X-100 detergent micelles was compared in the gel filtration assay, under conditions where ionic interactions should not contribute to association. Also here, (iv)-myr-TCKI behaved like TCKII (Figure 6). Interestingly, the two bands visible in TCKI preparations (Figure 6A) could be distinguished not only by virtue of their slightly different molecular weights but also by their distinct abilities to interact with Triton X-100 micelles (see fractions 17−22). The faintly visible upper band behaved like TCKII in the micelle association experiment (peak in fractions 18/19/20), while the more prominent lower band showed no visible displacement in the gel filtration profile (peak in fractions 20/21/22), eluting in the same place as TCKI or TCKII in the absence of liposomes or detergent micelles (data not shown).

Side-by-side comparison of TCKI and TCKII (data not shown) revealed that indeed the bulk of protein present in the two preparations differed slightly in molecular weight and that TCKII comigrated with the faint upper band visible in TCKI preparations (see Figure 6A). This difference in molecular weight is comparable to that seen between myristoylated and nonmyristoylated MARCKS (Manenti et al., 1993) or MRP (Vergeres et al., 1995), although in the latter case the nonmyristoylated protein migrates with slightly higher apparent molecular weight. Furthermore, about 10% of MARCKS isolated from bovine brain is reportedly nonmyristoylated (Manenti et al., 1993). This value is similar to the fraction of TCK activity present in the TCKI pool, which represents about 5% of the total TCK activity purified from sea urchin sperm tails (Quest & Shapiro, 1991a).

Functionally, TCKI preparations contain a fraction of about 20% of both TCK activity (Quest & Shapiro, 1991a) and protein (Figure 5A,B) that behaves in liposome and detergent micelle association experiments like TCKII, consistent with the notion that the upper band visible in TCKI preparations is TCKII (Figure 6A). The ability of TCKI to bind to PC/PS (75:25) liposomes is at least 25-fold reduced compared to TCKII (data not shown). These differences are similar to those reported between nonmyristoylated and myristoylated MARCKS (Manenti et al., 1993). Taken together, our data comparing TCKI and TCKII strongly suggest that (iv)-myr-TCKI is very similar if not identical to TCKII.

Myristoylation, in addition to mediating membrane association, may be an essential element contributing to the exclusive presence of TCK in the sea urchin sperm flagellum (Quest & Shapiro, 1991a, 1991b; Quest et al., 1992). The presence of both nonmyristoylated and myristoylated TCK variants in the enzyme preparations from sea urchin sperm tails would appear inconsistent with this notion, unless TCKI were derived from TCKII by demyristoylation, as has been reported for MARCKS (Manenti et al., 1994), rather than being synthesized directly as a nonmyristoylated TCK protein. Distinguishing between these possibilities may be experimentally feasible if TCK demyristoylation were not a constitutive event but occurred only upon sea urchin sperm activation.

A recent report analyzing the distribution of CK isozymes in chicken sperm has shown the ubiquitous mitochondrial CK isoform (Mi_a-CK) to be present in the expected location at the head−midpiece boundary, as well as at the distal end of the sperm tail. Likewise, the sarcomeric isoform Mi_b-CK is present along the entire sperm tail in addition to being present in the midpiece region (Kaldis et al., 1996). Interestingly, the latter MiCK isoform also binds efficiently to acidic phospholipids (Rojo et al., 1991), but, unlike TCK, lacks a myristoylation consensus sequence (Muehlebach et al., 1994). Results presented previously (Quest & Shapiro, 1991a) and here indicate that ionic forces, in addition to myristoylation, are involved in TCK−phospholipid interactions. Thus, as for MiCKs, elements within the CK repeats of TCK may contribute to its specific localization along the sperm flagellum, perhaps by providing additional lipid interaction sites or by mediating specific protein−protein interactions. Currently, studies are underway to assess precisely which TCK elements are involved in liposome binding and how liposome composition affects TCK association. Additionally, transfection experiments in polarized cells may yield insights concerning the role of myristoylation in targetting TCK to distinct membrane domains.

CONCLUSIONS

The experiments reported here address several important points concerning TCK. First, TCKII, the major TCK fraction purified from sea urchin sperm tails, was shown by GC/MS analysis and selective ion monitoring to contain myristoylglycine and is therefore a myristoylated protein. Second, TCKI, a minor fraction isolated from sea urchin sperm tails and characterized in the same manner, was found to be a nonmyristoylated sea urchin sperm tail creatine kinase. Third, TCKI contained all the sequence requirements for efficient NMT-dependent *in vitro* myristoylation, while in the same experiments TCKII was not significantly

myristoylated. Fourth, myristoylation appeared necessary and sufficient to convert TCKI into a protein that associates with PC/PS liposomes and Triton X-100 detergent micelles in the same manner as TCKII. These results are consistent with the notion that TCKI represents a fraction of TCKII that was either never myristoylated or demyristoylated, perhaps following sperm activation.

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REFERENCES

Alland, L., Peseckis, S. M., Atherton, R. E., Berthiaume, L., & Resh, M. D. (1994) *J. Biol. Chem. 269*, 16701−16705.
Blackshear, P. J. (1993) *J. Biol. Chem. 268*, 1501−1504.
Cadwallader, K. A., Paterson, H., Macdonald, S. G., & Hancock, J. F. (1994) *Mol. Cell. Biol. 14*, 4722−4730.
da Silva, A. M., & Klein, C. (1990) *J. Cell Biol. 111*, 401−407.
Goddard, C., & Felsted, R. L. (1988) *Biochem. J. 253*, 839−843.
Goddard, C., Arnold, S. T., & Felsted, R. L. (1989) *J. Biol. Chem. 264*, 15173−15176.
Hedo, J. A., Collier, E., & Wilkinson, A. (1987) *J. Biol. Chem. 262*, 954−957.
Herberg, F. W., Bell, S. M., & Taylor, S. S. (1993) *Protein Eng. 6*, 771−777.
Jones, T. L. Z., Simonds, W. F., Merendino, J. J. J., Brann, M. R., & Spiegel, A. M. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 568−572.
Kaldis, P., Stolz, M., Wyss, M., Zanolla, E., Rothen-Ruthishauser, B., Vorherr, T., & Wallimann, T. (1996) *J. Cell Sci. 109*, 2079−2088.
Kim, J., Shishido, T., Jiang, X., Aderem, A., & McLaughlin, S. (1994) *J. Biol. Chem. 269*, 28214−28219.
Koegl, M., Zlatkine, P., Ley, S. C., Courtneidge, S. A., & Magee, A. I. (1994) *Biochem. J. 303*, 749−753.
Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335−341.
Manenti, S., Sorokine, O., Van Dorsselaer, A., & Taniguchi, H. (1993) *J. Biol. Chem. 268*, 6878−6881.
Manenti, S., Sorokine, O., Van Dorsselaer, A., & Taniguchi, H. (1994) *J. Biol. Chem. 269*, 8309−8313.
McIlhinney, R. A. J. (1992) in *Lipid modifications of proteins: A practical approach* (Harper, N. M., & Trevor, A. J., Eds.) pp 15−35, Oxford University Press, New York.
McIlhinney, R. A. J., & McGlone, K. (1990) *J. Neurochem. 54*, 110−117.
McIlhinney, R. A. J., & Harvey, D. J. (1995) *J. Mass Spectrom. 30*, 900−910.
McIlhinney, R. A. J., Patel, P. B., & McGlone, K. (1994) *Eur. J. Biochem. 222*, 137−146.
Muehlebach, S. M., Gross, M., Wirz, T., Wallimann, T., Perriard, J.-C., & Wyss, M. (1994) *Mol. Cell. Biochem. 133/134*, 245−262.
Mumby, S. M., Heukeroth, R. O., Gordon, J. I., & Gilman, A. G. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 728−732.
Muszbek, L., & Laposata, M. (1993) *J. Biol. Chem. 268*, 8251−8255.
Pillai, S., & Baltimore, D. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7654−7658.
Quest, A. F. G., & Shapiro, B. M. (1991a) *J. Biol. Chem. 266*, 19803−19811.
Quest, A. F. G., & Shapiro, B. M. (1991b) in *Comparative spermatology 20 years after* (Bacetti, B., Ed.) pp 533−538, Raven Press, New York.
Quest, A. F. G., Chadwick, J. K., Wothe, D. D., McIlhinney, R. A. J., & Shapiro, B. M. (1992) *J. Biol. Chem. 267*, 15080−15085.
Quest, A. F. G., Bardes, E. S. G., & Bell, R. M. (1994) *J. Biol. Chem. 269*, 2953−2960.
Resh, M. D. (1989) *Cell 58*, 281−286.
Resh, M. D. (1994) *Cell 76*, 411−413.
Resh, M. D., & Ling, H. P. (1990) *Nature 346*, 84−86.
Rojo, M., Hovius, R., Demel, R. A., Nicolay, K., & Wallimann, T. (1991) *J. Biol. Chem. 266*, 20290−20295.
Schlieff, E., Schmitz, A., McIlhinney, R. A. J., Manenti, S., & Vergeres, G. (1996) *J. Biol. Chem. 271*, 26794−26802.
Silverman, L., & Resh, M. D. (1992) *J. Cell Biol. 119*, 415−425.
Stevenson, F. T., Bursten, S. L., Fanton, C., Locksley, R. M., & Lovett, D. H. (1992) *J. Exp. Med. 176*, 1053−1062.
Stevenson, F. T., Bursten, S. L., Fanton, C., Locksley, R. M., & Lovett, D. H. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7245−7249.
Tombes, R. M., & Shapiro, B. M. (1985) *Cell 41*, 325−334.
Towler, D. A., Gordon, J. I., Adams, S. P., & Glaser, L. (1988) *Annu. Rev. Biochem. 57*, 69−99.
Vergeres, G., Manenti, S., Weber, T., & Sturzinger, C. (1995) *J. Biol. Chem. 270*, 19879−19887.
Wilcox, C., Hu, J.-S., & Olson, E. N. (1987) *Science 238*, 1275−1278.
Wothe, D. D., Charbonneau, H., & Shapiro, B. M. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5203−5207.

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