The basis for the unusual properties of the catalytic subunit (C) of ram sperm cAMP-dependent protein kinase was investigated. Ram sperm C was purified and found by mass spectrometry (MS) to be ~890 Da smaller than C₀, the predominant somatic isoform. Partial internal amino acid sequence from ram sperm C was an exact match to that of bovine C₀, but differed from the predicted sequences for the Cₘ and Cᵧ isoforms. MS analysis of 2-nitro-5-thiocyanatobenzoic acid fragments showed that the mass difference originated in the amino-terminal region. A unique blocked amino-terminal fragment was isolated from sperm C and sequenced by a combination of tandem mass spectrometry and Edman degradation of a subfragment. The results revealed that the amino-terminal myristate and the first 14 amino acids of C₀ are replaced by an amino-terminal acetate and six different amino acids in sperm C. The predicted mass difference due to these changes is 899 Da. The region of homology between sperm C and C₀ begins at the exon 1/exon 2 boundary in C₀, suggesting that sperm C results from use of an alternate exon 1 in the C₀ gene. The different amino terminus of sperm C may be related to a unique requirement for localization of the “free” C subunit within the sperm flagellum.

The cAMP-dependent protein kinase (PKA) is a major enzyme in cellular signal transduction and is thought to mediate many of the physiological responses to cAMP in eukaryotic cells (1). Below a cAMP threshold concentration, PKA exists as an inactive tetramer of two catalytic and two regulatory subunits (CR₂C). The two R subunits form a dimer with each protomer inactive tetramer of two catalytic and two regulatory subunits (1). Below a cAMP threshold concentration, PKA exists as an inactive tetramer, releasing C to phosphorylate its substrates (for reviews, see Refs. 1 and 5–8).

Cyclic AMP-dependent signaling has an important role in the control of sperm movement. Mammalian sperm are nonmotile in the testis, but as they pass through the epididymis they acquire the capacity for motility. This process is known as “epididymal maturation” and is essential for the sperm to fertilize an egg (9). Several types of studies have shown that changes in sperm cAMP levels are involved in epididymal maturation (see Refs. 10–12 for reviews). The most direct evidence for a role of CAMP in the acquisition of the capacity for motility has come from studies of demembranated, reactivated sperm (13–15). When caudal epididymal or ejaculated sperm were demembranated by treatment with nonionic detergents, and then placed in an appropriate solution containing ATP, they were reactivated with a waveform very similar to that of intact ejaculated sperm. In contrast, under the same conditions demembranated testicular sperm exhibited very poor motility. However, if cAMP was added to the reactivation medium, the demembranated testicular sperm began to beat with a motility similar to that of the mature sperm models. Cyclic AMP-dependent motility also could be demonstrated in the demembranated ejaculated sperm if the sperm were metabolically inhibited to reduce their motility prior to demembranation (16, 17). The requirement for cAMP could be bypassed by addition of exogenous C to the reactivation solution, confirming that CAMP was acting via PKA (17). Therefore, cAMP-dependent phosphorylation is critical for sperm motility.

Sperm C (Cₚ) appears to have unusual solubility properties. It is generally accepted that, in the presence of cAMP, C is soluble in the cytoplasm (1, 18). However, in a previous study we observed that neither detergent nor cAMP alone released C activity from demembranated sperm, but that cAMP plus nonionic detergent did release the activity (17). These results suggested that Cₚ is bound to internal sperm structures by two types of bonds, one sensitive to detergent and one sensitive to cAMP.

There are at least three different isoforms of mammalian C, Cₐ, Cₜ, and Cᵧ, which are the products of different genes. Cₐ occurs in a wide variety of tissues (19); Cₜ also is widely distributed but is expressed in lesser amounts than Cₐ in most tissues except the brain (19, 20). Cᵧ appears to be expressed only in testis (21). Cₚ appears to be the predominant isoform in mammalian germ cells (22). Splice variants of both Cₐ and Cₜ also are known (23–25).

We now have further characterized Cₚ and investigated its relationship to the previously known isoforms of C. We found that Cₚ is the major, and perhaps the only, PKA catalytic subunit in sperm. We isolated Cₚ from ram sperm, which could be obtained in amounts sufficient for protein biochemistry.
Catalytic Subunit of Sperm PKA Has Unique Amino Terminus

Purification also was aided by the solubility properties of the subunit, which made it possible to obtain a homogeneous preparation of Cα by a simple two-step protocol. Mass spectrometry (MS) revealed that the mass of Cα was 890 Da less than that of Cβ from ovine striated muscle. The amino terminus of Cα was blocked, but partial amino acid sequence from internal tryptic and CNBr fragments was an exact match to the sequence of bovine Cα. Cα clearly differed in sequence from the Cβ and Cγ isoforms. An amino-terminal endoproteinase lysine-C fragment was isolated from Cα and sequenced by a combination of tandem mass spectrometry (MS/MS) and Edman degradation of an endoproteinase aspartate-N subfragment. The results revealed that the amino-terminal 14 amino acids and amino-terminal myristate of Cα are replaced by six different amino acids and an amino-terminal acetate in Cα. The predicted mass difference due to this replacement is 899 Da, in excellent agreement with the MS data. The region of homology between Cα and Cβ begins at exactly the exon 1/exon 2 boundary in Cα, suggesting that Cα results from the use of an alternate exon 1 in the Cα gene. The shorter amino terminus and lack of a myristate moiety may expose a hydrophobic portion of the catalytic core of Cα, allowing Cα to bind to a hydrophobic site within the flagellum and thus accounting for the unusual solubility properties of the subunit.

MATERIALS AND METHODS

Materials and used and their sources were: 4-aminobenzamidine, cAMP, CNBr, 3,5-dimethoxy-4-hydroxycinnamic acid, and NTLC from Aldrich; 14C-methylated protein molecular weight markers were from Amersham; Aquapore RP-300 C8 column (0.1 × 25 mm), Aquapore OD-300 C8 column (1 × 100 mm), and C18, 300 A column (0.5 × 150 mm) from Applied Biosystems; Tween 20 and SDS from Bio-Rad; endoproteinase Glu-C from Roche; nitrocellulose BA 83, 0.2 μm, from Millipore; [3H-32P]ATP from Amersham; Bovine Cα subunit, which made it possible to obtain a homogeneous preparation.

Collection of Ovine Sperm

Ejaculated sperm were collected and washed as described earlier (26). Epididymal and testis were obtained from a freshly killed ram. Epididymal sperm was collected by retrograde extrusion of the epididymis with wash buffer (27). The extruded sperm were then suspended in a Triton X-100/NaCl buffer (5 mM potassium phosphate, pH 6.5, 0.5%, v/v, Triton X-100, 150 mM NaCl, 1 mM EDTA, 25 μM leupeptin, 1 mM DTT), at a concentration of 3 × 10^8 flagella/ml. This treatment removed the plasma membrane and most of the soluble flagellar proteins that otherwise would coexist with Cα upon subsequent treatment with cAMP; Cα itself remained bound to the demembranated flagella.

Isolation of the PKA Catalytic Subunit (Cα) from Ovine Sperm Flagella

Extraction of Cα from Sperm Flagella by cAMP—Extraction was done at 4 °C. Sperm flagella (in PBSI) were centrifuged (17,500 × g, 15 min) and resuspended for 30 min in a Triton X-100/NaCl buffer (5 mM potassium phosphate, pH 6.5, 0.5%, v/v, Triton X-100, 150 mM NaCl, 1 mM EDTA, 25 μM leupeptin, 1 mM DTT), at a concentration of 3 × 10^8 flagella/ml. This treatment removed the plasma membrane and most of the soluble flagellar proteins that otherwise would coexist with Cα upon subsequent treatment with cAMP; Cα itself remained bound to the demembranated flagella. The suspension was then centrifuged (17,500 × g, 20 min), the supernatant discarded, and the pellet dispersed in KPNELD wash buffer (5 mM potassium phosphate, pH 6.5, 50 mM
NaCl, 1 mM EDTA, 25 μM leupeptin, 1 mM DTT, 0.22 mL of buffer/10^8 flagella) and centrifuged (1750 × g, 10 min). This was repeated with 0.167 mL of buffer/10^8 flagella. The washed pellet was then extracted for 20 min with KPNELD + 10 μM CaMP, 0.167 mL/10^8 flagella. The CaMP extract, which contained C_s, was transferred to a polypropylene tube that was treated with Triton X-100 to minimize nonspecific binding of C_s and then centrifuged at 27,000 × g for 15 min to remove residual flagella.

**Fast Protein Liquid Chromatography of CaMP Extract of Sperm Flagella**—The CaMP extract was made 0.2% (w/v) in β-octylglucoside and applied at 0.3 mL/min flow rate to a CM Fast Flow column (0.5 × 5 cm) previously equilibrated with chilled buffer A (20 mM potassium phosphate, pH 6.5, 1 mM EDTA, 50 mM NaCl, 0.1%, w/v, β- octylglucoside, 1 mM DTT). The column was washed with buffer A until the absorbance, which rose due to cAMP in the extract, returned to base-line. The column was eluted with 7 mL of a cold linear NaCl gradient of 0 to 160 and 280 mM NaCl. The column was eluted with a linear NaCl gradient (buffer A plus buffer B, total volume 150 mL) previously equilibrated with chilled buffer A (20 mM potassium phosphate, pH 6.5, 1 mM EDTA, 300 mM NaCl, 0.1%, w/v, β- octylglucoside, 1 mM DTT) at 0.2 mL/min flow rate. C_s was detected in fractions between 180 and 215 mM NaCl. Yield was typically 20–25 μg/6 mL of semen. β-Octylglucoside was included in the buffers to prevent nonspecific binding of C_s to the glass column, tubings, and tubes, as well as to stabilize the native conformation of C_s (31).

**Isolation of the PKA Catalytic Subunit (C_m) from Ovine Skeletal Muscle**

The procedure was adapted from Okuno and Fujisawa (31) for the isolation of bovine heart C. Working at 4 °C, about 600 to 800 g of skeletal muscle tissue from the hind legs and back of a ram were stripped of fat and connective tissue and then passed through a meat grinder (coarse setting). The ground tissue was mixed with 1.5 liters of homogenization buffer (10 mM potassium phosphate, pH 6.8, 1 mM EDTA, 0.1% DTT) and further processed to a smooth consistency in a Waring blender. The homogenized mass was then centrifuged at 18,000 × g for 30 min and the supernatant was collected, filtered through glass wool, and applied to a packed DE52 column (5 × 15 cm) equilibrated with the homogenization buffer. About 8 liters of wash buffer (55 mM potassium phosphate, pH 6.8, 1 mM EDTA, 15% glycerol, 5% SDS, 0.003% bromphenol blue) was added to the column, followed by about 2 liters of the DE52 elution buffer (45 mM potassium phosphate, pH 6.8, 0.1% EDTA, 1 mM DTT, 0.1%, w/v, Tween 20). The PKA homodimers bound to the DE52 resin; C_m was released by washing with DE52 equilibration buffer containing 100 μM CaMP. DE52 sequestered CaMP, so that C_m was eluted only after about twice the bed volume of the elution buffer was applied. The fractions containing C_m were identified by SDS-PAGE, and then applied to a CM Fast Flow column (1 × 8.5 cm, equilibrated with DE52 equilibration buffer). The column was washed with 20 mM potassium phosphate, pH 6.8, 1 mM EDTA, 50 mM NaCl, 0.1% (w/v) β- octylglucoside, 1 mM DTT and then subjected to a linear salt gradient of 50 mM to 300 mM NaCl (total volume of gradient, 50 mL; flow rate 0.4 mL/min). C_m eluted between 160 and 280 mM NaCl. The resulting C_m preparation was about 95% pure, with a yield of about 400 μg of C_m/100 g of skeletal muscle. It was made 40% in glycerol and stored at −20 °C. Homogeneous C_m was obtained by applying an aliquot of the glycerol-stabilized preparation (2–3 mL, 40–60 μg of C_m) to a Source 15G column (0.5 × 5 cm), equilibrated with buffer A and eluting with a linear NaCl gradient (buffer A plus buffer B, total volume of 7 mL, flow rate 0.4 mL/min). C_m eluted as a sharp peak between 250 and 265 mM.

**HPLC and Protein Sequencing**

All HPLC separations were carried out on a modified Hewlett-Packard HP1090 n system equipped with a UV photodiode array detector with a 1.7-μL microflow cell. For 20 μL/min flow rates an LC packing and Automatic Microflow splitter was used. Automated Edman degradations were performed on an Applied Biosystems/Perkin-Elmer 494 Procise sequencing system.

**Mass Spectrometry**

MALDI TOF MS was performed on a Perseptive Biosystems linear Voyager BioSpectrometry Workstation. Electrospray MS/MS was performed using a Perkin-Elmer Sciex API 365 benchtop triple quadrupole mass spectrometer equipped with MicroIonSpray and the BioTool-Box™ software package. Product ion MS/MS experiments were carried out using nitrogen as the dissociating gas at a collision cell pressure of 2.2 × 10⁻³ torr and a collision energy of 40 eV. Scans were obtained in the positive-ion mode from m/z 30 to 1500 with a step size of 0.25 atomic mass units and a dwell time of 0.75 ms. In order to increase sensitivity, the first MS was operated using low resolution (full width, half-height ~ 3 atomic mass units). Additionally, samples were infused at a flow rate of 200 nL/min using the MicrolonSpray source, thus allowing 140 scans to be signal averaged to improve signal-to-noise ratio.

**Trypsin Digestion of C_s**

C_s was blotted onto PVDF membrane as described above (see “Western Blotting”). The membrane was cut into small pieces (1 × 1 mm) and submerged under 50 μL of Digest Buffer (10% acetonitrile, 1% hydrogen Triton X-100, 100 mM ammonium bicarbonate, pH 8.2). One μg of trypsin in 4 μL of 50 mM acetic acid was added to the sample, which was then incubated overnight at 37 °C followed by direct injection of the supernatant onto the HPLC. Tryptic peptides were separated on a 1-mm × 25-mm Applied Biosystems (Aquapore RP-300) C_s column using a linear gradient from 100% solvent A (0.1% trifluoroacetic acid) to 55% solvent B (0.08% trifluoroacetic acid in acetonitrile/water: 70/30) in 30 min, then from 55% solvent B to 85% solvent B in 10 min at a flow rate of 150 μL/min. The eluent was monitored at 210 nm and fractions were collected manually.

**CNBr Cleavage of C_m and C_s at Methionyl Residues**

For electrophoretic analysis of fragments, about 2 μg each of C_m and C_s in 50 μL of buffer A were lyophilized. To each lyophilized sample was added 12.5 μL of 100 mM DTT, and the mixture then was allowed to stand at room temperature for 1 h. To start the cleavage reaction, 37.5 μL of 47 mM CNBr (in 88% formic acid) were added. The reaction mixture was incubated overnight (about 19 h) at room temperature in the dark. To stop the reaction, 100 μL of water was added and the resulting solution was lyophilized to remove the formic acid and unreacted CNBr. The pellet was resuspended in 100 μL of water and lyophilized, and afterward 15 μL of SDS sample buffer (50 mM Tris- HCl, pH 6.8, 15% glycerol, 5% SDS, 0.003% bromphenol blue) was added to the lyophilized sample. The sample was electrophoresed in a 0.75-mm thick 13% Tris-Tricine/SDS-polyacrylamide gel (32); the gel was silver stained to reveal the fragments.

**For sequencing of fragments, the starting sample contained about 10–15 μg of C. The cleavage products were separated by electrophoresis in a 1.5-mm gel and transferred to a PVDF membrane according to the protocol of Otter et al. (33), except that the transfer time was shortened from 17 to 12 h. The blot was stained with Amido Black and the fragments excised and sequenced.**

**NTCB Cleavage of C_m and C_s at Cysteinyl Residues**

The procedure was adapted from Jacobson et al. (34). The concentration of C_m and C_s (in fresh buffer A) was adjusted to 40 μg/mL and 50 μL of each was then lyophilized. The dried samples were redisolved in 100 μL of 10X denaturation buffer (50 mM Tris- HCl, pH 6.8, 6 M Urea, 100 mM DTT, 1% SDS) and then incubated overnight at 37 °C followed by direct injection of the lyophilized sample. The sample was electrophoresed in a 0.75-mm thick 13% Tris-Tricine/SDS-polyacrylamide gel (32); the gel was silver stained to reveal the fragments.

**Preparation of NTCB fragments for mass spectrometry was the same, except that 30 μg each of C_m and C_s were used as starting material. The Tris-Tricine gel was transferred to nitrocellulose (33) at 29°C for 5 min and then at 37°C for 20 min. The bands were revealed by staining with Ponceau S and then cut out for MALDI TOF MS.**

**Endoproteinase Lysine-C Digestion of C_m and C_s**

C_m and C_s were blotted on nitrocellulose which was then cut into 1 × 1-mm pieces and submersed under 50 μL of Digest Buffer. An aliquot of endoproteinase lysine-C (0.5 μg in 0.5 μL of 25 mM sodium phosphate, pH 7.5, 1 mM EDTA) was then added and the samples were incubated overnight at 37°C followed by direct injection of the supernatant onto
the HPLC. Endoproteinase lysine-C peptides were separated on a 0.5 × 150-mm Applied Biosystems column (C18, 300-A) using a linear gradient from 100% solvent A to 46% solvent B in 35 min, then from 46% solvent B to 60% solvent B in 10 min at a flow rate of 20 μl/min. The eluent was monitored at 210 nm and fractions collected manually.

Peptide Synthesis

Four peptides were synthesized in order to compare their MS/MS product ion spectra to the MS/MS product ion spectrum of the amino-terminally blocked endoproteinase lysine-C peptide derived from C: 1) acetyl-SANPNDVQEFLAK; 2) acetyl-ASNPDVKEFLAK; 3) acetyl-ASGPNRDVKEFLAK; and 4) acetyl-ASPPGDVKEFLAK. Peptides were synthesized on a Perkin-Elmer 432 A Synergy Peptide Synthesizer using HBTU activation and the 9-fluorenylmethoxycarbonyl protecting group strategy. Crude peptide mixtures were purified by reversed phase HPLC using an Aquapore OD-300 C18 column (1 × 100 mm) and a water/acetonitrile/trifluoroacetic acid gradient at 40 μl/min and 37 °C.

RESULTS

C, Has an Unusual Mobility in SDS-PAGE—Our previous results (17) showed that demembranated ram sperm models retained >90% of the sperm PKA activity; moreover, preparations of demembranated sperm with initially poor motility in the presence of cAMP became highly motile when cAMP was added, indicating that the cAMP-dependent pathway that initiates motility was still intact. To begin characterization of C, the presence of ATP became highly motile when cAMP was added, indicating that the cAMP-dependent pathway that initiates motility was still intact. To begin characterization of C, the presence of ATP became highly motile when cAMP was added, indicating that the cAMP-dependent pathway that initiates motility was still intact. To begin characterization of C, the presence of ATP became highly motile when cAMP was added, indicating that the cAMP-dependent pathway that initiates motility was still intact. 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Sperm. Two prominent bands at ~M, 40,000 and 39,000 migrated slightly faster than porcine C (~M, 41,000). To determine if either of these was C, the incubation with [γ-32P]ATP was carried out in the presence of PKI(5–24), a potent inhibitor of C (35), including ram C (17) activity. The inhibitor completely and specifically blocked the labeling of porcine C and the ~M, 40,000 band from sperm (Fig. 1B), indicating that the latter is likely to represent C. Labeling of the other bands was not affected by PKI(5–24). Interestingly, labeling of the prominent band at ~M, 69,000 was enhanced when the blocking reagent was the tyrosine kinase substrate poly(Glu,Tyr) instead of bovine serum albumin (results not shown), suggesting that this band represents a tyrosine kinase. Tyrosine kinases also have been implicated in the control of sperm motility (36, 37).

To confirm that the sperm protein kinase inhibited by
When purified ram Csm and ram sperm flagella were mixed together, two distinct, nonmerging bands were observed. These results clearly indicated that the unusual mobility of Cs is due to processing during sperm maturation. Previously we reported that demembranation of ram sperm with Triton X-100 (0.2%, 0.2 ml per 1.5 × 10⁸ sperm, 35 s) did not release significant PKI(5–24)-inhibitable protein kinase activity, and that subsequent extraction of the demembranated sperm with cAMP (100 μM, 5 min) did not release the activity, but that demembranation with Triton X-100 in the presence of cAMP solubilized ~50% of the activity (17). Unfortunately, the resulting extract also contained a large number of other proteins that complicated purification of Cs. More recently, we have found that Cs can be removed from purified sperm flagella in a near homogeneous state by a two-step procedure consisting of longer extraction with Triton X-100 (0.5%, 5 ml per 1.5 × 10⁸ sperm, 30 min) in the presence of 150 mM NaCl to remove the detergent- and salt-soluble proteins, followed by extraction with cAMP (10 μM, 20 min) in the absence of detergent to remove Cs. Apparently, the longer extraction with Triton X-100 disrupted bonds that impeded the release ofCs under our previous extraction conditions. Inclusion of 150 mM NaCl in the Triton X-100 extraction caused release at this step of some proteins that otherwise would be removed by the cAMP buffer and thus contaminate the Cs. Fig. 3, TX-100, shows the flagellar proteins released by the Triton X-100/NaCl extraction. After two washes with buffer (washes), the flagella were exposed to 10 μM cAMP, which removed nearly pure Cs from the flagella (cAex). In some preparations, this cAMP extract also contained small amounts of a 20-kDa protein (results not shown). This apparently was not a proteolytic fragment of Cs, as it was not recognized by polyclonal antibodies to Cs (results not shown). Cs was further purified by passing the cAMP extract through a CM Fast Flow column (Fig. 3, lanes marked 10–21). This removed any trace of the 20-kDa protein, which eluted later than Cs. Using this procedure, 20–25 μg of purified Cs could be obtained from ~6 ml of semen (~10¹⁰ sperm).

PKI(5–24) is the catalytic subunit of PKA, the proteins of demembranated ram sperm were probed in Western blots with a polyclonal antibody against bovine C (Fig. 2A). The antibody reacted with a single sperm protein that migrated slightly faster than porcine C in the SDS-polyacrylamide gels. This protein could be extracted from sperm flagella by treatment with cAMP in the presence of Triton X-100 (Fig. 2A), in agreement with our previous observation that much of the PKI(5–24)-inhibitable protein kinase activity could be released from sperm by this same treatment (17). These results provided independent evidence that the ~M₄₀,₀₀₀ band from sperm is an isoform of C.

**C is a Tissue Variant**—The unusual mobility of ram Cs could have been due to species (porcine versus ovine) or tissue (somatic versus sperm) variation. To clarify this, ram skeletal muscle C (Csm) was partially purified and compared directly to the sperm isoform (Fig. 2B). Because the predominant isoform of C in skeletal muscle is Cs (19), the isoform that we purified from muscle is presumed to be predominantly or entirely Cs. The skeletal muscle subunit had the same mobility as porcine C, and both migrated slightly slower than the sperm subunit. When purified ram Csm and ram sperm flagella were mixed together, two distinct, nonmerging bands were observed. These results clearly indicated that the unusual mobility of Cs is tissue specific.

**Cs is Contained Primarily in Flagella**—The relative distribution of Cs in demembranated, isolated sperm heads and tails was also investigated. The vast majority of Cs was located in the flagella (Fig. 2B, lanes H and F). Because the predominant isoform of C in skeletal muscle is Cs (19), the isoform that we purified from muscle is presumed to be predominantly or entirely Cs. The skeletal muscle subunit had the same mobility as porcine C, and both migrated slightly slower than the sperm subunit. When purified ram Csm and ram sperm flagella were mixed together, two distinct, nonmerging bands were observed. These results clearly indicated that the unusual mobility of Cs is tissue specific.

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**Cs is Contained Primarily in Flagella**—The relative distribution of Cs in demembranated, isolated sperm heads and tails was also investigated. The vast majority of Cs was located in the flagella (Fig. 2B, lanes H and F); note that lane H was loaded with 3 × 10⁶ sperm heads versus 1 × 10⁶ flagella in lane F); however, some Cs was detectable in the sperm heads. Similar results were obtained with intact sperm heads and intact sperm tails (results not shown). These results are consistent with previous reports that PKA is located primarily in the sperm flagella (38–43).

The Unusual Mobility of Cs is Not the Result of Epididymal Processing—Some sperm proteins undergo processing during epididymal maturation (44–46). To determine if such processing was responsible for the unusual mobility of Cs, sperm were isolated from the testis and regions of the epididymis, and the relative mobilities of their PKA catalytic subunits were compared in Western blots. Fig. 2C shows a Western blot of C, from demembranated ram testicular sperm, demembranated epididymal sperm (cauda, corpus, and caput), and demembranated ejaculated sperm flagella. The mobility of Cs was identical in sperm from all stages, and in all cases was slightly faster than that of somatic C. These results indicate that the apparently smaller size of Cs is not due to processing during sperm maturation.

**Purification of Cs**—To understand the difference between Cs and Csm, it was necessary to purify Cs for structural character-
dicted for bovine somatic Ca with a myristylated glycine at the amino terminus (40,858 Da) (see below).

Because Cs is smaller than Csm, the difference in mass between the two subunits cannot be due to the addition of an unusual post-translational modification to Cs. Somatic Ca, which Csm is presumed to be, has been extensively characterized. Its major post-translational modifications are myristylation of the amino-terminal glycine (47), which adds 210 Da to the mass predicted by sequence alone, and phosphorylation at Thr-197 and Ser-338 (48), each of which would add 80 Da to the predicted mass. Therefore, even the total absence of these post-translational modifications in Cs could not account for the difference in mass. By the process of elimination, the difference must arise from differences in the primary structures of the two proteins. It seemed unlikely that this difference was due simply to proteolysis during fractionation of the sperm or purification of Cs, because: 1) no band was ever observed in sperm that migrated with somatic Ca, and 2) attempts to obtain amino-terminal amino acid sequence from Cs were unsuccessful, suggesting that its amino terminus was blocked (see below).

The Partial Amino Acid Sequence of Cs Is Identical to That of Cg—Beebe et al. (21) reported the isolation and sequencing of human cDNA clones encoding an unusual tissue-specific isoform of Ca, which they termed Cg. Cg mRNA was found in detectable levels only in testis. Cg has not yet been isolated from testis, but when it was expressed and purified from transfected cells it migrated in SDS-PAGE at 39–40 kDa versus 41–42 kDa for Ca (49). Although Cg reportedly is not sensitive to PKI, its expression in testis and the similarity in its apparent mass to that of Cs raised the possibility that Cs might be Cg. Human Cg and Ca differ at 74 amino acid residues, suggesting that the ovine homologs of these two isoforms should be distinguishable readily by even partial amino acid sequences. Attempts to obtain amino-terminal sequence directly from the intact ram Cs were unsuccessful, suggesting that its amino terminus is blocked. Consequently, purified C s was digested with trypsin to generate tryptic fragments, and the fragments purified by HPLC. Similarly, purified ram Cs or Csm was cleaved with CNBr, and the larger fragments separated by SDS-PAGE and transferred to PVDF membrane. Selected tryptic and CNBr fragments were then sequenced by automated Edman degradation. Fig. 5 shows the resulting amino acid sequences compared with similar sequences predicted for bovine Ca and Cb, and human Ca and Cy. There is no published sequence for ovine Ca or Cb. However, the ovine Cg sequence exactly matched that of bovine Ca (78 out of 78 residues). Moreover, in 17 out of 18 positions where human Ca differed from human Cg, ovine Cs was identical to human Ca. Therefore, Cs is not Cg. Similarly, ovine Cs was identical to bovine Ca at 5 out of 5 positions where bovine Ca differed from bovine Cg, indicating that Cs is not Cg. These results strongly suggested that Cs is a short variant of Ca.

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FIG. 5. Partial sequence of Cs and Csm from CNBr and tryptic fragments. Sequences obtained for ram Cs and Csm fragments are aligned with the corresponding fragments from bovine Ca and Cb, and human Ca and Cy. Residues different from those of Cs and Csm are highlighted.

FIG. 6. NTCB fragments from Cs and Csm. Panel A, Tris-Tricine gel of products resulting from NTCB cleavage of ram Cs (Cs) and Csm (Csm). The three bands are intact Cs (C) and fragments 1 (1) and 2 (2). Starting material was 2 μg each of Cs and Csm. Positions of molecular weight markers (M) are indicated. Panel B, MALDI TOF mass spectra of fragments 1 and 2 of Cs and Csm. Singly, doubly, and triply charged ion peaks are evident, suggesting that its amino terminus was blocked (see below).

The Partial Amino Acid Sequence of Cs Is Identical to That of Ca—Beebe et al. (21) reported the isolation and sequencing of human cDNA clones encoding an unusual tissue-specific isoform of C, which they termed Cy. Cy mRNA was found in detectable levels only in testis. Cy has not yet been isolated from testis, but when it was expressed and purified from transfected cells it migrated in SDS-PAGE at 39–40 kDa versus 41–42 kDa for Ca (49). Although Cy reportedly is not sensitive to PKI, its expression in testis and the similarity in its apparent mass to that of Cs raised the possibility that Cs might be Cy. Human Cy and Ca differ at 74 amino acid residues, suggesting that the ovine homologs of these two isoforms should be distinguishable readily by even partial amino acid sequences. Attempts to obtain amino-terminal sequence directly from the intact ram Cs were unsuccessful, suggesting that its amino terminus is blocked. Consequently, purified Cs was digested with trypsin to generate tryptic fragments, and the fragments purified by HPLC. Similarly, purified ram Cs or Csm was cleaved with CNBr, and the larger fragments separated by SDS-PAGE and transferred to PVDF membrane. Selected tryptic and CNBr fragments were then sequenced by automated Edman degradation. Fig. 5 shows the resulting amino acid sequences compared with similar sequences predicted for bovine Ca and Cb, and human Ca and Cy. There is no published sequence for ovine Ca or Cb. However, the ovine Cg sequence exactly matched that of bovine Ca (78 out of 78 residues). Moreover, in 17 out of 18 positions where human Ca differed from human Cg, ovine Cs was identical to human Ca. Therefore, Cs is not Cg. Similarly, ovine Cs was identical to bovine Ca at 5 out of 5 positions where bovine Ca differed from bovine Cg, indicating that Cs is not Cg. These results strongly suggested that Cs is a short variant of Ca.
A single CNBr fragment of C sm was analyzed and found to have a sequence identical to that of bovine Cα (Fig. 5). Although this sequence is inadequate to distinguish between Cα and Cβ, the results are consistent with our presumption that C sm is the conventional Cα isoform.

Localization of the Region of Mass Difference—To delimit the regions that are different between C s and C sm, the purified subunits were treated with NTCB, which cleaves at cysteinyl residues. There are only 2 cysteinyl residues (Cys-199 and Cys-343) out of a total of 350 amino acids in either bovine or human Cα. The three fragments resulting from a complete cleavage of Cα by NTCB are predicted to have masses of 0.9 kDa (residues 343–350), 16.6 kDa (residues 199–342), and 23.0 kDa (residues 1–198). When the NTCB fragments of C s and C sm were electrophoresed in a Tris-Tricine/SDS-polyacrylamide gel, three major bands were seen (Fig. 6A). The largest band corresponded to the intact polypeptide. Based on the predicted sizes of the fragments, fragment 1 must be the amino-terminal fragment, and fragment 2 must correspond to residues 199–342. Fragment 2 from C s and fragment 2 from C sm had identical mobilities, whereas fragment 1 of C s migrated more rapidly than fragment 1 of C sm. The fragments were then transferred to nitrocellulose and analyzed by MALDI TOF MS (Fig. 6B). Fragment 2 of C s and fragment 2 of C sm had nearly identical masses of 17,970 and 17,967 Da, respectively. In contrast, fragment 1 of C s had a mass of ~23,620 Da, whereas fragment 1 of C sm had a mass of ~24,444 Da, a difference of ~824 Da. Because this difference is similar to the difference in masses between the intact polypeptides (~890 Da), most of the difference in mass must be due to structural differences in the amino-terminal halves of the proteins. Substantial sequence in this part of C s (residues 30–45, 72–91 and 129–133; see Fig. 5) already had been found to match the sequence of Cα, so these regions could be ruled out as being the source of the difference.

Identification of a Unique Endoproteinase Lysine-C Fragment from C s—There are 34 lysyl residues in bovine Cα, of which 8 occur in the first 59 residues. It therefore seemed likely that digestion of C s and C sm with endoproteinase lysine-C would allow the detection of any dissimilar fragments. Fig. 7A shows HPLC chromatograms of endoproteinase lysine-C fragments from C s and C sm. A prominent peak eluting at 26 min was observed in the C s digest but not in the C sm digest. MALDI TOF MS analysis of this peak indicated that it contained a single peptide with a mass of 1474 Da (results not shown). Similarly, a peak at 1475 Da was observed in a MALDI TOF mass spectrum of the endoproteinase lysine-C digest of C s, but not in the C sm digest (Fig. 7B). An attempt to determine the amino-terminal sequence of the 1474-Da peptide obtained by HPLC was unsuccessful, suggesting that its amino terminus was blocked. These results indicated that this fragment probably represented the amino terminus of C s.

Amino Acid Sequence of a Unique Amino-terminal Fragment from C s—The structure of the 1474-Da peptide was solved by a combination of MS/MS on a triple quadrupole mass spectrometer and Edman sequence analysis of an endoproteinase aspartate-N cleavage product.

In product ion MS/MS, precursor ions (or parent ions) of a particular m/z value are selected in the first quadrupole (Q1) of a triple quadrupole mass spectrometer and allowed to enter the second quadrupole (Q2). The second quadrupole acts as a collision cell and is filled with a neutral gas (in this case nitrogen). The parent ions undergo fragmentation through collisions with this neutral gas, a process called collisionally activated dissociation, or CAD. These product ions (or daughter ions) are then analyzed in the third quadrupole (Q3). For peptide ions, fragmentation specifically at the amide bonds results
in a series of ions with charge retention on either the carboxyl terminus ("y-ions") or amino terminus ("b-ions"). The sequence of the peptide can be deduced from these, as well as other fragment ions in the daughter ion spectrum.

The doubly charged ion (m/z 738.3) was selected and fragmented, producing the spectrum shown in Fig. 8B. Initial interpretation of the spectrum confirmed an amino-terminal blocked residue (acetyl-AS) and provided much of the carboxyl-terminal sequence (DV(K/Q)EF(I/L)AK). Leucine and isoleucine have identical masses and lysine and glutamine have nearly identical masses and so could not be distinguished. Because only a single aspartate was detected in the sequence, the 1474-Da peptide was cleaved with endoproteinase aspartate-N, and the fragments then sequenced by Edman degradation. As predicted, a single Edman sequence (DVKEFLAK) was obtained. This indicated that the residue following valine was lysine and confirmed the rest of the carboxyl-terminal sequence. Determination of the amino-terminal sequence of the 1474-Da fragment was complicated because the data were consistent with either glycine-glycine or asparagine on either side of the proline; glycine-glycine and asparagine have identical masses. To resolve this uncertainty, a series of synthetic peptides were made containing permutations of asparagine and glycine-glycine. The tandem mass spectrum of only one of these peptides, acetyl-ASNPNDVKEFLAK, was virtually identical to that of the 1474-Da amino-terminal peptide isolated from Cα (cf. Fig. 8, A and B), indicating that this is the correct sequence.

Although the 1474-Da peptide was derived from an endoproteinase lysine-C digest, the appearance of an internal lysine in the fragment is not completely unexpected because this lysine is followed by a glutamic acid residue. Previous literature has suggested that this enzyme may be hindered at glutamic acid residues (50).

**Fig. 8.** Analysis of a unique 1474-Da peptide by tandem mass spectrometry. MS/MS product ion spectra for the doubly charged ion with m/z 738.5 from the synthetic peptide acetyl-ASNPNDVKEFLAK (Panel A), and the precursor-ion with m/z 738.5 of the 1474-Da amino-terminally blocked peptide derived from the endoproteinase lysine-C digest of Cα (Panel B). The two spectra are in excellent agreement with regard to fragment ions observed and their relative intensities. As would be expected from a peptide with a carboxyl-terminal lysine, a strong y-ion series is observed; doubly charged fragment ions are prominent due to the internal lysine. Peaks due to cleavage at the amino-terminal amide bond of the proline residue are particularly prominent, as has been reported by others (59). Ions marked with an asterisk (*) correspond to fragment ions originating from a contaminant Triton X-100 precursor ion with a nominal mass of 740 Da. Because Q1 was operated in a low resolution mode, some ions with m/z 740 were admitted into the collision cell for fragmentation. In a subsequent set of experiments the resolution on Q1 was increased in order to prohibit the contaminant Triton X-100 precursor ions from entering the collision cell. Peptide MS/MS product ion spectra obtained under these conditions did not contain the "*" fragment ions, whereas high resolution MS/MS product ion scans for m/z 740 (i.e. prohibiting fragmentation of m/z 738.5 peptide ions) did produce the "*" contaminant ions.

**Fig. 9.** Amino acid sequences of amino-terminal regions of ovine Cα and bovine Cα. Residues 1–6 of Cα and 1–14 of bovine Cα are highlighted. The position of the exon 1/exon 2 boundary in mouse Cα is indicated by brackets.
A comparison of this sequence to the amino-terminal portion of bovine Ca (Fig. 9) showed that residues 7–13 of Ca (VKE-FLAK) are identical to residues 15–21 of Cs. Residues 1–6 of Cs are completely different from residues 1–14 of Ca. The calculated mass difference between the amino-terminal sequence of bovine Ca (including an amino-terminal myristyl group) and that of Cs (including an amino-terminal acetyl group) is 899.2 Da, in excellent agreement with the difference in mass determined by MALDI TOF MS.

**DISCUSSION**

The PKA catalytic subunit of ram sperm (Cs) is unusual in its solubility properties (17) and its electrophoretic mobility in SDS-PAGE (see Figs. 1 and 2). The solubility properties were utilized as a critical first step in a simple purification protocol that permitted the isolation of ram sperm Cs in amounts sufficient for characterization by peptide mapping, protein sequencing, and mass spectrometry. The results showed that ovine Cs is identical to bovine Ca in 85/85 amino acids sequenced between Ca residues 15 and 317, but that the amino-terminal 14 amino acids of Ca have been replaced with 6 amino acids of completely different sequence in Cs. Moreover, the amino terminus of Cs is acetylated, rather than myristylated as in Ca. As a result, Cs is predicted to be 899 Da smaller than Ca, in excellent agreement with the mass difference (~890 Da) determined by MS. This difference undoubtedly accounts for the more rapid mobility of Cs in SDS-PAGE.

A closer inspection of the amino-terminal sequences of ovine Cs and bovine Ca reveals that the homology between the two subunits begins precisely at the site (Val-15 in Ca) of the exon 1/exon 2 junction in the mouse gene (51) (Fig. 9). Inasmuch as the residues carboxyl-terminal to this site that have been sequenced in Cs are an exact match to those of bovine Ca, it seems likely that Cs is a splice variant of Ca resulting from use of an alternative 5′ exon. If Cs and Ca were different genes, some differences in amino acid residues carboxyl-terminal to the exon 1/exon 2 boundary probably would have been detected in the tryptic and CNBr fragments that we sequenced. Öyen et al. (22) reported that a probe specific for Ca mRNA detected both 2.3- and 2.4-kilobase mRNAs in rat pachytime spermatocytes and round spermatids; the smaller band may have been Cs mRNA.

The position of the first intron is conserved between mouse Ca and Cβ subunits (51), and two isoforms of bovine Cβ have been identified that similarly appear to use alternative versions of exon 1 (24). Different amino termini resulting from alternative promoter use associated with two different 5′ exons also have been reported for the neuronal PKA catalytic subunit of *Aplysia* (52). It has been suggested that the different amino termini may be important for targeting specific isoforms of C to specific sites in the cell (Refs. 24 and 52, and see below).

A mouse C pseudogene, termed Cx, has been cloned (53). It does not possess intron sequences and, based on Northern blot analysis and RNase protection assays of testis and brain mRNA, is not expressed, indicating that it is a retroposon. It was mapped to the mouse X chromosome, whereas Cs, which it most closely resembles, maps to chromosome 8. Compared with the Ca sequence, the Cx sequence contains frameshift deletions that give rise to numerous termination codons in the reading frame. The nucleotide sequence of Cx is homologous to that of Ca downstream of the exon 1/exon 2 junction, but is completely different upstream of this boundary. Cummings et al. (53) reasoned that this apparent truncation at the splice site for the first intron in Cs occurred because the mRNA intermediate for Cx was incompletely spliced. However, the predicted amino-terminal sequence for Cx is MPSSSNDV-7, which is very similar to the amino-terminal sequence (M)ASNPNDV- of ovine Cs. Thus, it seems more likely that the Cx pseudogene arose by reverse transcription of an intact mouse Cs mRNA.

Beebe et al. (21) reported the cloning of cDNAs encoding a unique testis-specific isoform of C, which they termed Cy. Cy has not been isolated from testis, but when expressed in transformed adrenal cells, it migrated faster than Cs in SDS-PAGE (49). It also differed from Ca in substrate specificity and sensitivity to PKI(5–24). Comparison of the partial sequence of ovine Cs with that predicted for human Cy showed that Cs is not Cy. However, in our protein kinase blots, we detected a sperm protein kinase migrating slightly faster than Cs that was not inhibited by PKI(5–24). Cy cross-reacts weakly with an anti-bovine C polyclonal antibody (49), and although we did not observe any cross-reactivity with the faster, PKI-insensitive protein kinase band using the same antibody, the possibility remains that this band represents ovine Cy. If so, it may be possible to isolate Cy directly from ram sperm and conduct studies of its structure and properties.

In the course of cloning Cy from the human testis cDNA library, Beebe and colleagues (21) also isolated Cs clones, but they did not obtain a clone encoding the amino-terminal part of the molecule. Apparently, this was because *Eco*RI was used to excise the Cy cDNA inserts, and an internal *Eco*RI site was present at the codons for Glu-17 and Phe-18 of the human Ca gene (54). Because there was complete identity between their truncated cDNA sequence and the sequence of a Ca cDNA from a HeLa cell library (54), Beebe et al. (21) did not screen further for the 5′ end of the testis Ca cDNA. It is possible that Cs is present in human testis, but was undetected because all clones characterized were missing exon 1 and a portion of exon 2.

The substitution of the short acetylated amino-terminal segment in Cs for the amino-terminal myristyl moiety and first 14 residues of somatic Cs is likely to have important consequences for the structure of the subunit. In Cs, this segment, which corresponds to exon 1 and is termed the “myristylated motif,” forms the first two turns of a long amphipathic α helix, the A-helix, that extends across both the small and large lobes of the catalytic core (residues 42–297), then turns sharply inward to terminate with the myristyl group occupying a deep hydrophobic pocket (55). The myristate is tightly anchored by interactions with hydrophobic groups from four parts of the molecule that are widely separated in the linear sequence. The myristate in turn anchors one end of the A-helix, which covers a large and mostly hydrophobic portion of the catalytic core. Bacterially expressed recombinant Cs lacking the myristyl group is more heat labile than the myristylated subunit (56), and a deletion mutant lacking the myristyl group and residues 1–14 also has decreased thermal stability (57). These results indicate that the myristylated motif is important for stabilizing the protein. Interestingly, deletion of the myristylated motif did not affect the catalytic properties of the recombinant Cs. Therefore, Cs might be expected to be less thermodynamically stable than Cs, but to be similar with regard to catalytic activity. In crystals of the non myristylated recombinant Cs, the amino-terminal 14 residues were not visible, indicating that they are unstructured in the absence of the myristyl group (58). It would be of interest to determine if the short amino-terminal domain of Cs is similarly unstructured.

In Cs the amino acid residues of the myristylation motif and the rest of the A-helix shield the myristate and hydrophobic surface of the catalytic core from the aqueous environment, with the result that free Cs is soluble. It is possible that replacement of the myristylation motif with a shorter stretch of residues, coupled with replacement of the myristate with an acetate, would leave a portion of the hydrophobic core exposed.
in C\textsubscript{a}. Additionally, because the shorter A-helix would be more loosely held to the hydrophobic core as a consequence of the absence of the myristyl anchor, even more of the hydrophobic surface beneath it might be exposed. In this case, the surface of C\textsubscript{a} would have a hydrophobic patch that might cause it to bind to other hydrophobic surfaces in the flagellum.

Indeed, such a hydrophobic site may exist on C\textsubscript{a}. In the course of developing our purification protocol for C\textsubscript{s}, we observed that C\textsubscript{s} eluted from a Source 15S column as a broad peak (results not shown). This was in contrast to C\textsubscript{sm} which eluted as a sharp peak. Source 15S is an anion exchanger with a polystyrene/divinylbenzene matrix. Two possible explanations for the different elution profiles are that either the C\textsubscript{s} sample was heterogeneous, or there was a substantial nonspecific interaction of the matrix with C\textsubscript{s} but not with C\textsubscript{sm}. The former is unlikely, because only one band was observed in silver-stained SDS-PAGE and only one peak was detected in mass spectra of purified C\textsubscript{s}. The broad peak observed with C\textsubscript{s} was more likely due to hydrophobic interaction with the column matrix. The corollary of this is that C\textsubscript{s} has an exposed hydrophobic domain not present in C\textsubscript{sm} (C\textsubscript{a}).

The presence of a hydrophobic patch on C\textsubscript{a} could explain why cAMP alone could not release C\textsubscript{s} from demembranated sperm (17), but C\textsubscript{s} was released from sperm in the presence of cAMP plus Triton X-100 (17) or by cAMP following extended extraction with Triton X-100 (this report). The Triton X-100 may disrupt hydrophobic interactions between C\textsubscript{s} and a flagellar protein other than R. Consistent with this, when unmyristylated recombinant C\textsubscript{a} was co-crystallized with a detergent, the hydrophobic pocket was found to be occupied by a molecule of the detergent (55). This raises the possibility that the unusual amino terminus of C\textsubscript{s} may be related to a unique requirement for localization of the “free” subunit to a specific compartment within the highly ordered sperm tail.

Many proteins are potential substrates for phosphorylation by C. In the sperm, phosphorylation of specific proteins by C may be achieved by controlling access of C to its potential substrates. For example, if C were tethered to a flagellar structure by its unique amino terminus or a hydrophobic patch, it would be unable to diffuse freely upon cAMP-induced release from R, and could phosphorylate only those substrates within its immediate vicinity. Such anchoring would prevent promiscuous phosphorylation of incorrect substrates, with its potentially deleterious effects on flagellar motility. Alternatively, anchoring of C\textsubscript{s} may keep the “free” subunit from sterically interfering with the motile machinery of the axoneme.

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The Catalytic Subunit of the cAMP-dependent Protein Kinase of Ovine Sperm Flagella Has a Unique Amino-terminal Sequence
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