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# Human stanniocalcin: A possible hormonal regulator of mineral metabolism

HENRIK S. OLSEN*†, MARIO A. CEPEDA*, QING-QING ZHANG*, CRAIG A. ROSEN*, BENITO L. VOZZOLO‡, AND GRAHAM F. WAGNER‡

*Human Genome Sciences, 9620 Medical Center Drive, Rockville, MD 20850-3338; and ‡Department of Physiology, Faculty of Medicine, University of Western Ontario, London ON, Canada N6A 5C1

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**ABSTRACT** **We have isolated a human cDNA clone encoding the mammalian homolog of stanniocalcin (STC), a calcium- and phosphate-regulating hormone that was first described in fishes where it functions in preventing hypercalcemia. STC has a unique amino acid sequence and, until now, has remained one of the few polypeptide hormones never described in higher vertebrates. Human STC (hSTC) was found to be 247 amino acids long and to share 73% amino acid sequence similarity with fish STC. Polyclonal antibodies to recombinant hSTC localized to a distinct cell type in the nephron tubule, suggesting kidney as a possible site of synthesis. Recombinant hSTC inhibited the gill transport of calcium when administered to fish and stimulated renal phosphate reabsorption in the rat. The evidence suggests that mammalian STC, like its piscine counterpart, is a regulator of mineral homeostasis.**

Stanniocalcin (STC) is a calcium-regulating hormone in bony fishes that has never been described in higher vertebrates, including mammals (1). The hormone is synthesized by the corpuscles of stannius (CS), endocrine glands that are associated with the kidneys of all fishes with a bony skeleton (2). The primary function of STC in fishes is the prevention of hypercalcemia and a rise in serum calcium levels is the primary stimulus for secretion (3). Upon release into the circulation, STC lowers calcium transport by the gills thereby reducing its rate of influx from the environment into the extracellular compartment (1). A second equally important action of STC is stimulation of phosphate reabsorption by renal proximal tubules (4). The consequence of this renal effect is increased levels of plasma phosphate, the latter of which combines with excess calcium and promotes its disposal into bone and scales. Because the CS have never been identified in higher vertebrates, it has long been assumed that STC was unique to fishes. However, recent evidence of STC immunoreactivity in human kidney and serum argues for a more widespread existence of the hormone (5).

By a process of random sequencing of human tissue cDNAs, we have isolated a lung-derived cDNA clone whose deduced protein sequence bears a strikingly high level of homology to salmon and eel STC (6, 7).§ Data indicating that human (h)STC inhibits calcium uptake in fish and phosphate excretion in rats suggest that hSTC is a hormonal regulator of mineral metabolism.

## MATERIALS AND METHODS

**cDNA Isolation and Analysis.** *cDNA isolation.* The initial expressed sequence tag (EST) clones used in the study were discovered by scientists at The Institute for Genomic Research by using established EST methods (8, 9). These clones were part of a larger EST project (10). This clone was used for rescreening the same library and a full-length clone encoding hSTC was obtained.

*Southern blot analysis.* Ten-microgram aliquots of human genomic DNA were cut with *Bam*HI, *Eco*RI, and *Xba* I and then separated on an 0.8% agarose gel. After transfer to a nylon membrane, the blot was probed with $^{32}$P-labeled hSTC cDNA and washed under high stringency.

*Northern blot analysis.* RNA was separated in a 0.8% agarose/formaldehyde gel in 1× Mops buffer (10× Mops = 0.2 M Mops/50 mM NaOAc/10 mM EDTA). After transfer to a nylon membrane, the blot was probed with $^{32}$P-labeled hSTC cDNA and washed under high stringency, and the RNA expression pattern was analyzed by autoradiography.

**Expression of hSTC in Bacteria.** hSTC was cloned into the bacterial expression (pQE) vector and a histidine-tagged protein was purified by ion-affinity column chromotography according to the manufacturer (Quiagen, Chatsworth, CA).

Briefly, the bacterial culture harboring the hSTC expression plasmid was grown to an $OD_{600}$ of 0.4, induced with 1 mM isopropyl β-D-thiogalactoside, and grown for an additional 4 h. After induction, the cell pellet was dissolved in 6 M guanidine hydrochloride (pH 8.0) and applied to the ion-affinity column. After extensive washing, the protein was renatured by applying a gradient of urea (4 M to 0 M) in 150 mM NaCl/10% (vol/vol) glycerol. Protein was eluded in 250 mM imidazole/150 mM NaCl/25 mM Tris/10% glycerol and dialyzed.

**Fish Bioassay.** For testing the effects of hSTC in fishes, an established fish STC bioassay was employed essentially as described (1). This bioassay monitors the inhibitory effects of STC on gill calcium transport. Goldfish (1.2 ± 0.1 g per 10 fish per group) were given two intraperitoneal injections, 1 h apart, of recombinant hSTC, purified salmon STC, or saline, and then placed in tanks of water containing $^{45}$Ca (100,000 dpm/ml) for a 3-h period. The fish were then sacrificed and ashed overnight in a muffle furnace, and the isotope content of the resulting ash was determined by scintillation counting. The rate of gill calcium transport in each fish was determined on the basis of body isotope content and water-specific activity and was expressed as μmol of $Ca^{2+}$ per kg (body weight) per h. Individual treatment groups were considered significantly different than solvent-injected controls if $P < 0.05$ (ANOVA and Dunnet's test).

**Immunocytochemistry.** Antibodies were prepared in rabbits after three monthly immunizations, each of 200 μg of bacterially expressed recombinant hSTC dissolved in Freund's adjuvant/saline, 1:1 (vol/vol). The development of antibody titer was monitored by ELISA and the highest titer was obtained after the third immunization. Western blot analysis of

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Abbreviations: STC, stanniocalcin; h, human; ICC, immunocytochemistry; CS, corpuscles of stannius; STCir, STC immunoreactive.

†To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U46768).

human kidney extracts and recombinant hSTC yielded two closely spaced bands in both instances. Fresh human kidney biopsies were obtained from Surgical Pathology, University Hospital, London, with patient consent, and immediately fixed overnight in PBS containing 4% (wt/vol) paraformaldehyde (pH 7.4). Tissues were dehydrated, embedded in paraffin, and 4-$\mu$m serial sections were mounted on coated slides. For immunocytochemistry (ICC), tissue sections were dewaxed, rehydrated, and equilibrated in ICC diluent buffer (0.025 M Tris, pH 7.5/0.15 M NaCl). Sections were then treated for 10 min with undiluted normal goat serum to reduce background staining prior to an overnight incubation with a 1:1000 dilution of hSTC antiserum. After an extensive wash in ICC buffer, the sections were incubated for 1 h in peroxidase-coupled goat anti-rabbit IgG. The sites of antibody binding were visualized with 0.025% diaminobenzidene prepared in ICC buffer containing 0.01% $H_2O_2$. After development, the sections were counterstained in hematoxylin, dehydrated, and mounted. Control procedures included the application of normal rabbit serum or hSTC antiserum preabsorbed with hSTC in lieu of antiserum alone.

**Rat Bioassay.** Recombinant hSTC was tested for effects in rats by using standard methods for assessment of renal function. Male Wistar rats (300 g) were anesthetized with Inactin (100 mg/kg) and ketamine (10 mg/kg), and a tracheostomy was performed to facilitate breathing. Body temperature was maintained at 37 ± 0.5°C with a heating pad. A right jugular vein cannula was used for the infusion of saline and test substances. A right femoral artery cannula was used for monitoring mean arterial pressure and for blood sampling. Both ureters were cannulated near the renal pelvis for urine collection. The animals were infused with saline for 1 h after surgery prior to commencing an experiment. To start an experiment, urine was collected over the first 20-min interval, C1, and a blood sample was taken at the midpoint of urine collection. Recombinant hSTC or solvent was given as a 500-$\mu$l bolus 15 min into the second collection period, C2. Urine and blood were then collected over the next 80 min, C3 through C6. At the end of the experiment, the animals were sacrificed and the kidneys were removed, decapsulated, and weighed. Urine volume was calculated gravimetrically and expressed as ml per min per g (kidney weight). Mean arterial pressure was determined for each clearance period by averaging the readings over each clearance period. Plasma and urinary concentrations of $Na^+$ and $K^+$ were determined by flame photometry, whereas calcium (11) and inorganic phosphate (12) were determined colorimetrically. On the basis of these analyses, electrolyte excretion rates in clearance periods C3 through C6 were expressed as changes from C1 levels. The data were analyzed by repeated measures ANOVA and groups were considered significantly different if $P < 0.05$.

## RESULTS AND DISCUSSION

A partial cDNA clone with sequence similarity to salmon STC (6) was isolated by random sequencing of an early-stage human fetal lung cDNA library. This clone was used for rescreening the same library and a full-length clone encoding hSTC was obtained. The human cDNA encodes a protein that is 247 amino acids long (Fig. 1) and the level of sequence similarity when compared to salmon STC (6) was 92% over the first 204 amino acids, of which 118 residues were identical (Fig. 2). The last 43 residues on the C-terminal end of hSTC were completely divergent. However, this has also proven to be the case among fish species (6) and suggests that the C-terminal end is not critical for biological activity. Several features are conserved between fish STC and hSTC. For instance, the 11 half cystines in the mature protein core that participate in inter- and intrachain disulfide bonding occupy the same positions in salmon STC (6) and hSTC. This implies that the human protein

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MLQNSAVLLVLVISASATHEAEQNDSVSPRKSRV

AAQNSAEVVRCLNSALQVGCGAFACLENSTCDTD

GMYDICKSFLYSAAKFDTQGKAFVKESLKCIANG

VTSKVFLAIRRCSTFQRMIAEVQEECYSKLNVCS

IAKRNPEAITEVVQLPNHFSNRYYNRLVRSLLEC

DEDTVSTIRDSLMEKIGPNMASLFHILQTDHCAQ

THPRADFNRRRTNEPQKLKVLLRNLRGEEDSPSH

IKRTSHESA
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FIG. 1. Deduced amino acid sequence of hSTC. A partial cDNA clone with sequence similarity to salmon STC (6) was isolated by random sequencing of an early-stage human fetal lung cDNA library. Commencing with an initiator methionine and terminating with a stop codon, the human cDNA encodes a protein that is 247 amino acid residues long. The glycosylation consensus sequence is underlined.

is a homodimer in the native state as in the case of salmon. Salmon STC contains an additional half cystine in the hydrophobic leader sequence, however, that is not conserved in the human protein (6). The glycosylation consensus sequence Asn-Ser-Thr has also been conserved (6, 7, 13), indicating that hSTC is glycosylated like its piscine counterpart. Indeed, baculovirus-expressed hSTC binds readily to the plant lectin, concanavalin A, a feature that has been exploited in purifying the recombinant glycosylated protein (14, 15). In salmon, pro-STC is cleaved between Arg-33 and Phe-34 to yield a 223-residue mature protein core with phenylalanine on the N terminus (6). We have not yet established the correct cleavage site for mature human STC.

To assess the biological effects of hSTC, the bacterially expressed protein was injected into fish and rodents. For these studies, the entire open reading frame of the cDNA clone was placed in a bacterial expression vector containing a histidine tag sequence and the expressed protein was purified on a metal-ion affinity column. The ability of hSTC to mimic the fish hormone was verified in an established bioassay that measures the inhibitory effects of STC on gill calcium transport in fishes (1). In response to intraperitoneal injections of both hSTC (10 mg/kg) and salmon STC (1 mg/kg), gill calcium transport in the goldfish was significantly reduced in comparison to saline-injected controls (Fig. 3). This indicated that hSTC was indeed bioactive and capable of substituting for the fish hormone, presumably by binding to the fish STC receptor.

Recent findings suggest that the purified fish hormone has limited effects, if any, on calcium metabolism in mammals, particularly the parameters affected by parathyroid hormone such as serum calcium, bone resorption, and urinary cAMP (16). Salmon STC does, however, promote phosphate reabsorption by the fish kidney (4), which is an indirect mechanism for the lowering of plasma calcium levels. With this in mind, the effects of recombinant hSTC on phosphate reabsorption were assessed in anesthetized male rats by using standard clearance procedures for estimating renal function (17). In response to a single bolus injection [5 nmol of hSTC per kg (body weight)], phosphate excretion was significantly decreased in the rat (Fig. 4) with no concomitant effects on other plasma and urinary electrolytes or renal function. A similar effect was seen with baculovirus-expressed hSTC. Hence, recombinant hSTC also proved capable of mimicking an established effect of STC in fishes when tested in a mammalian model system.

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                                  10        20        30
Human                    MLQNSAVLLVLVISASATHEAEQNDSVSPRKSRVAAQN
                                 : ||:::|| ::::::: |||::| ::::
Salmon                   MLAKFGLCAVFLVLGTAATFDTDPEEA-SPRRARFSSNS

 40        50        60        70        80        90
SAEVVRCLNSALQVGCGAFACLENSTCDTDGMYDICKSFLYSAAKFDTQGKAFVKESLKC
:::|:||||:||:||||:|||||||||||||:|||: |:::||:|:||||:|||||||:|
PSDVARCLNGALAVGCGTFACLENSTCDTDGMHDICQLFFHTAATFNTQGKTFVKESLRC
  7
100       110       120       130       140       150
IANGVTSKVFLAIRRCSTFQRMIAEVQEECYSKLNVCSIAKRNPEAITEVVQLPNHFSNR
|||||||||| :||||::|||||:|||||||||:|::|::|::|||||:||||:|:||:||
IANGVTSKVFQTIRRCGVFQRMISEVQEECYSRLDICGVARSNPEAIGEVVQVPAHFPNR

160       170       180       190       200       210
YYNRLVRSLLECDEDTVSTIRDSLMEKIGPNMASLFHILQTDHCAQTHPRADFNRRRTNE
||: |::|||:|||:||:::|::|::::||:|::||::||::||:|
YYSTLLQSLLACDEETVAVVRAGLVARLGPDMETLFQLLQNKHCPQGSNQGPNSAPAGWR

220       230       240
PQKLKVLLRNLRGEEDSPSHIKRTSHESA

WPMGSPPSFKIQPSMRGRDPTHLFARKRSVEALERVME
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FIG. 2. Amino acid sequence comparison of hSTC and salmon STC. A solid line between species denotes identity and double dots denote similarity. Note the high level of identity in the core of the molecule. The level of sequence similarity when compared to salmon STC (6) was 92% over the first 204 amino acids, of which 118 residues were identical. The underlined sequence denotes the glycosylation consensus sequence. The last 43 residues on the C-terminal end of hSTC were completely divergent from the salmon, as is the case between the different fish STCs (6, 7). Sequence alignment was done with the University of Wisconsin Genetics Computer Group TFASTA program (182).

The hSTC gene is present as a single copy according to Southern blot analysis. To identify its chromosomal locus, a genomic clone was isolated and used in fluorescent *in situ* hybridization as described (18). The analysis of chromosomal spreads indicated that the hSTC gene was localized to band 8p21. When the tissue distribution of STC gene expression was examined by Northern blot analysis, low levels of expression were detected in several tissues including kidney, bone marrow, and thymic stromal cells (Fig. 5). ICC (19) was employed to identify cellular sources of the protein in human kidney by using a polyclonal antiserum to recombinant hSTC. In the kidney, no immunoreactivity was detected in the glomeruli, vascular elements, or hematopoietic tissue surrounding the nephrons; STC-immunoreactive (STCir) cells were confined to the nephron, specifically in distal convoluted tubule and the collecting tubule. Some STCir cells in the collecting tubule were phenotypically unique due to their large size and ten-

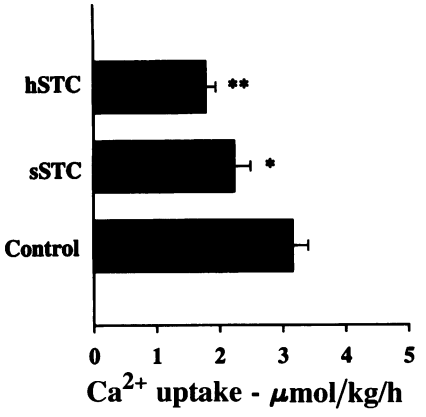


FIG. 3. hSTC inhibits gill calcium transport in fish. hSTC and salmon STC (sSTC) were tested for effects on gill calcium transport as described (1). Goldfish (1.2 ± 0.1 g; 10 fish per group) were given intraperitoneal injections of hSTC (10 mg/kg), salmon STC (1 mg/kg), or saline and placed in tanks of $^{45}Ca$-containing water (50,000 dpm/ml) for 3 h. The fish were then sacrificed and individually ashed overnight at 600°C, and the isotope content of the ash was determined by scintillation counting. Based on the body weight of the fish and the specific activity of the water, gill calcium transport in each fish was expressed as μmol of $Ca^{2+}$ per kg (body weight) per h. Both hSTC (**, $P < 0.01$) and salmon STC (*, $P < 0.05$) had statistically significant inhibitory effects on gill calcium transport (two-tailed ANOVA and Dunnet's test).

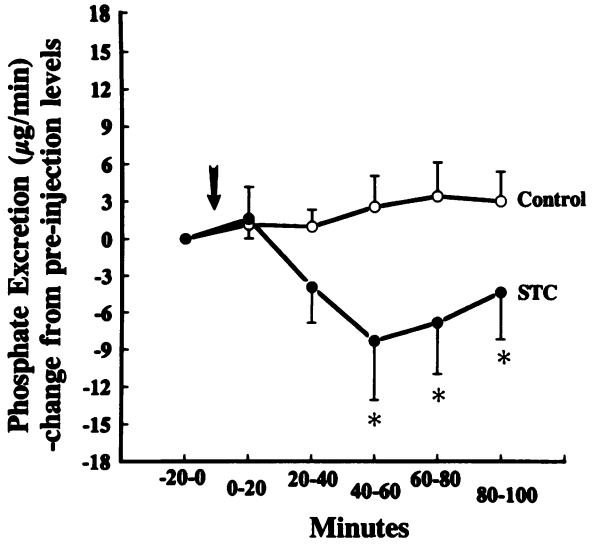


FIG. 4. hSTC inhibits phosphate excretion in the rat. Rats (250 ± 10 g; 5 rats per group) were maintained under Inactin anesthesia with catheters in one jugular vein, one carotid artery, and both ureters and were continuously infused with inulin and *p*-aminohippuric acid for measurement of glomerular filtration and renal blood flow, respectively, as described (15). Bolus injections of hSTC (5 nmol/kg) or saline were given via the jugular catheter after the first urine collection period (arrow) and renal function, plasma and urinary electrolytes ($Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$, and $PO_4$), and blood pressure were monitored throughout. Phosphate excretion was maximally inhibited by hSTC after 60 min and remained significantly different thereafter ($P < 0.01$; repeated measures ANOVA). STC had no effect on any other parameters.

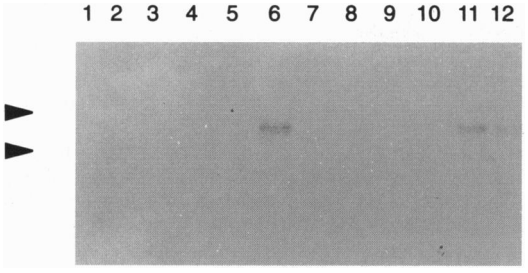


FIG. 5. Northern blot analysis of hSTC mRNA expression. The hSTC cDNA was radiolabeled and hybridized to 10 μg of total RNA unless otherwise indicated. A 1-week exposure of the blot is shown. Lanes: 1, stomach; 2, thymus; 3, spleen; 4, peripheral T cells; 5, leukocytes; 6, kidney; 7, lung; 8, liver; 9, heart; 10, pancreas; 11, thymic stromal cells (1.5 μg); 12, bone marrow [1 μg of poly(A) RNA]. The arrowheads at left denote the positions of 18S (bottom) and 28S (top) rRNAs.

dency to have multiple nuclei (Fig. 6*A*), suggesting that they were functionally different than surrounding cells. Antibody staining of STCir cells was abolished when the primary antiserum was preabsorbed with hSTC (Fig. 6*B*). In fishes, the CS glands are derived from the kidneys. Individual STC cells bud off from the nephron tubules during embryogenesis, coalesce to form CS glands, and then migrate to the kidney surface prior to hatching (20). In the most primitive bony fishes, however, the glands remain deep within the kidneys associated with individual nephrons (21). Therefore, the presence of STCir cells in the mammalian nephron is entirely consistent when viewed from an evolutionary perspective. The ICC results obtained in the present study with hSTC antiserum have confirmed and extended earlier ICC findings in human kidney using antibodies to the fish hormone. In the case of both antisera, staining was confined to the nephron tubule, as no staining was observed in glomeruli, proximal tubules, vascular elements, or hematopoietic tissue. However, whereas both antiserum stained distal tubule cells, the hSTC antiserum also stained cells in the thick ascending limb and collecting tubules. As our fish antiserum is highly crossreactive with recombinant hSTC, the different ICC staining patterns obtained with the two antiserum may be indicative of there being different STC cell types.

The hormonal regulation of mineral homeostasis in mammals is a complex process involving parathyroid hormone (22, 23), calcitonin (24), and the active metabolite of vitamin D (25). Parathyroid hormone and vitamin D counteract hypocalcemia by stimulating bone resorption, as well as intestinal and renal calcium transport, and parathyroid hormone also enhances renal phosphate excretion. Calcitonin, on the other hand, counteracts hypercalcemia by inhibiting osteoclastic bone resorption, although its importance as a minute-to-minute regulator of calcium homeostasis remains controversial (26). The complexity of mineral homeostasis is reflected in the numerous diseases associated with the impaired regulation of calcium and phosphate, which impact on renal function, and the vascular, neuronal, and muscular systems, in addition to bone mineralization. Recent findings involving tumor-induced osteomalacia in humans (27) and murine models of X chromosome-linked hypophosphatemia (28) imply there is room for additional humoral regulators of mineral homeostasis in mammals and STC warrants consideration as a candidate. Given the structural similarities between hSTC and fish STC and their comparable effects on renal function, STC may have the same role in mammals and fishes: preventing hypercalcemia, in part, through its stimulatory effects on phosphate reabsorption. The remarkable evolutionary conservation between fish and mammalian STC suggests an important role for this hormone in higher vertebrates.

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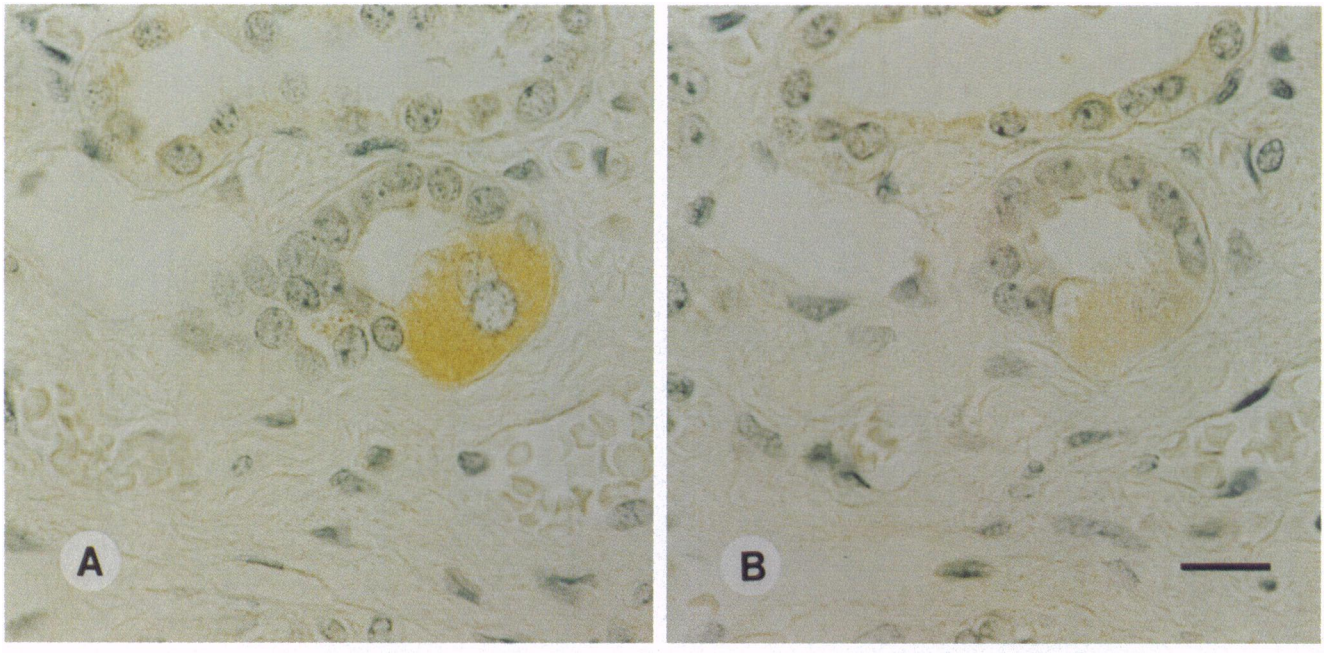


FIG. 6. STCir cells are present in human kidney nephron tubule. ICC was performed as described (19). Fresh biopsies of human kidney were fixed overnight in phosphate-buffered 4% (wt/vol) paraformaldehyde (pH 7.2), dehydrated, and embedded in paraffin. Dewaxed 5-μm serial sections were incubated overnight with a 1:1000 dilution of rabbit anti-hSTC serum in Tris-buffered saline (pH 7.5) (*A*) or the same antiserum dilution preabsorbed with recombinant hSTC (*B*). The slides were then washed for three 10-min periods in Tris-buffered saline, incubated for 30 min with peroxidase-coupled goat anti-rabbit IgG, washed as before, and developed in 0.025% diaminobenzidene containing 0.01% hydrogen peroxide. (*A*) Large multinucleated cell in the collecting tubule portion of the nephron in outer medullary kidney stained by the antiserum. (*B*) Adjacent section treated with preabsorbed antiserum and showing no specific staining. (Bar = 15 μm.)

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**Note Added in Proof.** While this manuscript was in review, a similar sequence was reported by Chang *et al.* (29).


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