

Characterization of Mammalian Stanniocalcin Receptors

MITOCHONDRIAL TARGETING OF LIGAND AND RECEPTOR FOR REGULATION OF CELLULAR METABOLISM*

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The polypeptide hormone stanniocalcin (STC) is widely expressed in mammalian tissues. STC acts locally in kidney and gut to modulate calcium and phosphate excretion, and its overexpression in mice results in high serum phosphate, dwarfism, and increased metabolic rate. STC has also been linked to cancer, pregnancy, lactation, angiogenesis, organogenesis, cerebral ischemia, and hypertonic stress. In this report we have characterized the STC receptor and the functional targeting of ligand and receptor to mitochondria. For receptor binding analysis, a stanniocalcin-alkaline phosphatase fusion protein was engineered. Subsequent binding assays using the fusion protein indicated that kidney and liver contained the highest number of binding sites with affinities of 0.8 and 0.25 nM, respectively. Intriguingly, purified mitochondria from both tissues yielded similar high affinity binding sites. Fractionation analysis revealed that the majority of binding sites were localized to the inner mitochondrial membrane. In further studies, we characterized the time course of STC-alkaline phosphatase fusion protein sequestration by intact mitochondria. *In situ* ligand binding also revealed discrete, displaceable binding to plasma membranes and mitochondria of nephron cells and liver hepatocytes. The existence of mitochondrial receptors prompted a similar search for the ligand. Immunogold electron microscopy revealed that STC was preferentially concentrated in the mitochondria of all nephron segments targeted by STC. Subcellular fractionation revealed that >90% of cellular STC immunoreactivity was mitochondrial, confined to the inner matrix, and similar in size to recombinant STC (50 kDa). In functional studies, recombinant STC had concentration-dependent stimulatory effects on electron transfer by sub-mitochondrial particles. Collectively the evidence implies a role for STC in cell metabolism.

A growing number of hormones act directly on subcellular structures to modulate cell function. Examples include parathyroid-related peptide, which directly targets the nucleus to exert anti-apoptotic (1) and mitogenic effects (2), and transforming growth factor β 1, which localizes to mitochondria where it is involved in preventing apoptosis through regulation

of membrane potential (3). In this study we demonstrate functional localization of stanniocalcin to mitochondria, suggesting that it should be added to this list.

Stanniocalcin (STC)¹ is a homodimeric glycoprotein hormone first discovered in fish and, more recently, in mammals. In both vertebrate classes STC is widely expressed and operates via local and endocrine signaling pathways (4–6). In fish, the endocrine form of STC controls calcium homeostasis through regulation of calcium/phosphate transport by the gills, gut, and kidney (7). Locally produced STC is operative in the fish gonad (8). In mammals, locally produced STC regulates renal and intestinal calcium and phosphate transport (6, 9, 10), whereas the ovaries produce an endocrine form of STC during pregnancy and lactation (11). STC has also been linked to a number of phenomena such as cancer (12–15), angiogenesis (16), organogenesis (17), cerebral ischemia (18), neurogenesis (19, 20), and hypertonic stress (21). Finally, STC overexpression in mice leads to high serum phosphate, dwarfism, and increased metabolic rate (22, 47). Despite this growing body of knowledge, little is known of STC signaling or STC receptors. In this report we have characterized mammalian STC receptors and described a novel mitochondrial-targeting pathway for both ligand and receptor.

EXPERIMENTAL PROCEDURES

STC Fusion Protein Production—An STC-alkaline phosphatase fusion protein was generated essentially as previously described (23). Briefly, the protein-coding region of the mouse STC cDNA was amplified by PCR to add restriction sites (*NheI* and *Bgl*II) and then ligated in-frame, upstream of the human placental alkaline phosphatase gene in the commercially available APTag vector (GenHunter) (Fig. 1A). After bacterial amplification, the vector was purified and sequenced to confirm the desired open reading frame.

The STC-AP expression vector was stably transfected into Madin-Darby canine kidney (MDCK) cells using ExGen 500 (Fermentas) and selected in 250 μ g/ml Zeocin (Invitrogen). As a control, the vector without insert (AP) was also stably transfected into MDCK cells as described above to generate secreted alkaline phosphatase. Media was harvested and concentrated before quantitating the alkaline phosphatase activity from STC-AP and AP-expressing cells as previously described (23). The STC content of STC-AP fusion protein media was quantified using a well characterized STC radioimmunoassay (24).

Receptor Binding Assays—Receptor binding assays were conducted on isolated nuclear, mitochondrial, and plasma membrane fractions from liver and kidney to obtain relative estimates of specific binding. Cell fractionation was performed as described (25). Briefly, tissues were minced with a razor blade and then Dounce-homogenized in 5 volumes of 0.1 M Tris-HCl, pH 7.6, containing 0.25 M sucrose and 5 mM EDTA. The homogenate was then centrifuged twice at 1,000 $\times g$ for 15 min, once at 15,000 $\times g$ for 30 min, and finally at 100,000 $\times g$ for 90 min, thereby isolating crude nuclear, mitochondrial, and plasma membrane

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¹ The abbreviations used are: STC, stanniocalcin; hSTC, human STC; RIA, radioimmunoassay; AP, alkaline phosphatase; MDCK cells, Madin-Darby canine kidney cells; CD cells, collecting ducts.

fractions, respectively. Subcellular fractions were resuspended in 50 mM HEPES, quantified for total protein, and stored at -70°C until use. High purity mitochondrial fractions were obtained by Percoll gradient centrifugation (26). The relative purity of each subcellular fraction was determined using 5'-nucleotidase, acid phosphatase, glucose-6-phosphatase, and succinate dehydrogenase assays as representative markers of plasma membranes, lysosomes, endoplasmic reticulum, and mitochondria as previously described (27).

For binding assays, 200- μg aliquots of each subcellular fraction were incubated with 0.5 nM STC-AP, STC-AP plus 1 $\mu\text{g}/\text{ml}$ purified STC, or the equivalent amount of secreted AP activity in Hanks' balanced salt solution containing 0.1% bovine serum albumin, pH 7.5 (HBHA), for 90 min at room temperature. To separate bound and free ligand, tubes were centrifuged for 3 min at $10,000 \times g$ to pellet the protein followed by two 1-ml washes of ice-cold HBHA. Washed pellets were solubilized in 200 μl of 1% Triton X-100 in 10 mM Tris-HCl, pH 8.0, for subsequent detection of AP activity (28).

The correlation between binding activity and protein concentration was established by adding 0.5 nM STC-AP to increasing amounts of purified liver membrane protein (100–500 μg). Membranes were incubated and washed, and AP activity was determined as described above. The pH dependence of binding was determined on 200- μg aliquots of membrane protein suspended in 50 mM acetate, HEPES, or Tris-based buffers to achieve pH ranges of 3–5, 6–7, and 8–10, respectively. To further establish the specificity of STC-AP binding, highly purified NIH hormone preparations were tested for their abilities to displace STC-AP binding. Binding of STC-AP was measured in the presence of each hormone (30 $\mu\text{g}/\text{ml}$).

Saturation binding assays were performed essentially as described above with increasing amounts of STC-AP, AP alone, or STC-AP + 1 μM purified recombinant human STC (hSTC) to obtain estimates of K_d and B_{max} . Additions were made to fixed amounts of membrane or mitochondrial protein (200–400 μg), and all samples were processed as above.

Sub-mitochondrial Localization of STC Receptors—To more precisely localize the sites of STC binding, we compared equal numbers of whole rat liver mitochondria and mitoplasts for receptor binding activity. Mitoplasts were prepared with 1% digitonin as described. To determine whether STC-AP was capable of being sequestered by intact mitochondria, we performed time course incubations of STC-AP (0.2 nM for 5, 15, 30, and 60 min) to 100- μg aliquots of freshly isolated mitochondria. After binding, proteinase K digestion (5 units/ml for 5 min on ice) was employed to destroy all externally bound ligand before the measurement of internalized STC-AP. Total, bound STC-AP activity was also quantified in undigested mitochondria as controls.

In Situ Ligand Binding Assays—To localize STC binding sites at the histological level, *in situ* ligand binding was performed as previously described (23), with the exception that paraffin-embedded sections were used instead of cryosections as the latter yielded only weak binding activity when tested. Paraformaldehyde-fixed, paraffin-embedded tissue sections were de-waxed, rehydrated, and equilibrated in HBHA before incubation with 0.5 nM STC-AP, AP, or STC-AP plus 1 μM hSTC in HBHA for 90 min at room temperature. Thereafter, the tissue sec-

tions were washed in HBHA with 0.1% Tween and fixed for 30 s in 60% acetone, 3% formaldehyde in 20 mM HEPES, pH 7.5. Endogenous AP activity was inactivated by a 60-min incubation in HBHA at 65°C . Color development of ligand-associated AP activity was then initiated at room temperature by the addition of AP detection buffer (100 mM Tris-HCl, pH 9.5, containing 100 mM NaCl, 5 mM MgCl_2 , 0.33 mg/ml nitro blue tetrazolium, and 0.17 mg/ml BCIP). Finally, slides were washed in ultrapure water to stop the reaction and mounted.

Subcellular Localization of STC Ligand—To localize STC at the subcellular level, rat liver, and kidney were Dounce-homogenized and fractionated according to Graham and Higgins (25) to yield nuclear (1,000 $\times g$), light mitochondrial (10,000 $\times g$), heavy mitochondrial (17,000 $\times g$), and plasma membrane fractions (100,000 $\times g$). The supernatant from the 100,000 $\times g$ spin was also saved to represent the cytosol. Serial dilutions of each fraction (200–800 μg) were then assessed for STC content by RIA. The data were expressed as ng of STC/mg of protein.

To more precisely localize STC within mitochondria, isolated liver mitochondria were subjected to either proteinase K and digitonin treatments. To determine whether STC was attached to the outer membrane, 200- μg aliquots of mitochondria were re-suspended in buffer (0.22 M mannitol, 0.07 M sucrose, 10 mM HEPES-KOH, pH 7.6, 1 mM MgCl_2 , 1 mM EDTA) with or without 5 units/ml proteinase K and incubated for 10 min on ice. Digested mitochondria were then washed twice to remove residual proteinase K, dissolved in 0.1% Triton X-100, and assayed by RIA (24) or Western blot. To test for the presence of STC in the inner mitochondrial matrix, mitoplasts (digitonin-treated mitochondria devoid of the outer membrane or intermembrane space) were generated by incubating intact mitochondria with 1% digitonin in the above buffer for 20 min on ice (29). Mitoplasts were then pelleted, dissolved in 0.1% Triton X-100, and assayed by RIA or Western blot. Controls in this case consisted of incubating equivalent amounts of mitochondria in buffer alone over the same time period.

Ultrastructural Localization of STC Ligand—To localize STC at the subcellular level, mouse kidneys were fixed in a mixture of 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 3 h and then embedded in Lowicryl K4M. Thin tissue sections were preincubated in 10 mM sodium phosphate buffer containing 150 mM sodium chloride, pH 7.4 (phosphate buffer) containing 0.1% skimmed milk powder and 50 mM glycine. The sections were then incubated for 1 h at room temperature with a 1:100 dilution of rabbit anti-hSTC in phosphate buffer containing 1% normal goat serum. Sites of primary antibody binding were then visualized with goat anti-rabbit IgG conjugated to 10-nm colloidal gold particles (Sigma) diluted 1:50 in phosphate buffer. Sections were stained with uranyl acetate before examination by electron microscopy.

Functional Assays of Stanniocalcin Using Sub-mitochondrial Particles—To determine the possible effects of STC on mitochondrial respiratory chain activity, biologically active recombinant hSTC (20) was used in combination with a Mitoscan kit according to the manufacturer's directions (Harvard Bioscience, Inc. Madison, WI). The kit utilizes bovine heart sub-mitochondrial particles to assess electron transfer

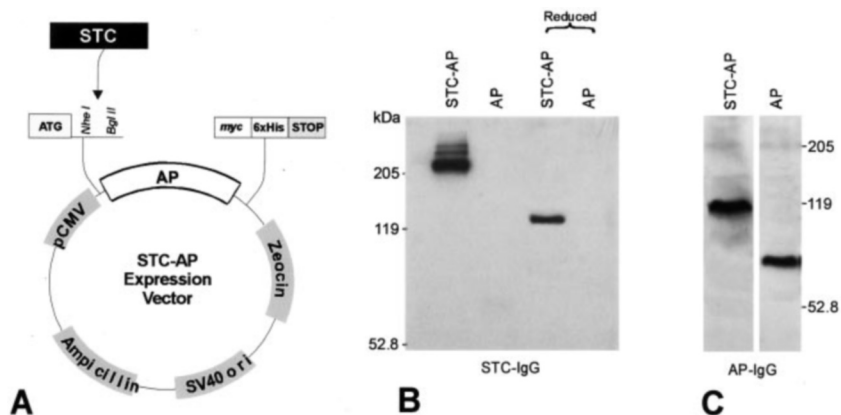


FIG. 1. Generation of stanniocalcin-alkaline phosphatase fusion protein. A, mammalian expression vector for STC-AP fusion protein. The plasmid contains a cytomegalovirus promoter (pCMV) for high expression in mammalian cells, a Zeocin resistance gene for selection of stable transfectants, and c-Myc and His₆ affinity tags for purification. Using PCR to add *NheI* and *BglII* restriction sites, the protein-coding region of the mSTC cDNA was inserted in-frame ahead of the human placental AP gene as indicated by the black box and arrows. The vector without insert was used for generation of secreted AP alone. B and C, Western blot of conditioned media from MDCK STC-AP- and AP-stable transfectants. Media was separated by PAGE on 7.5% gels and probed with an anti-STC antibody under non-reducing and reducing conditions ($\pm 5\%$ β -mercaptoethanol) (B) or with an anti-AP antibody under reducing conditions (C).

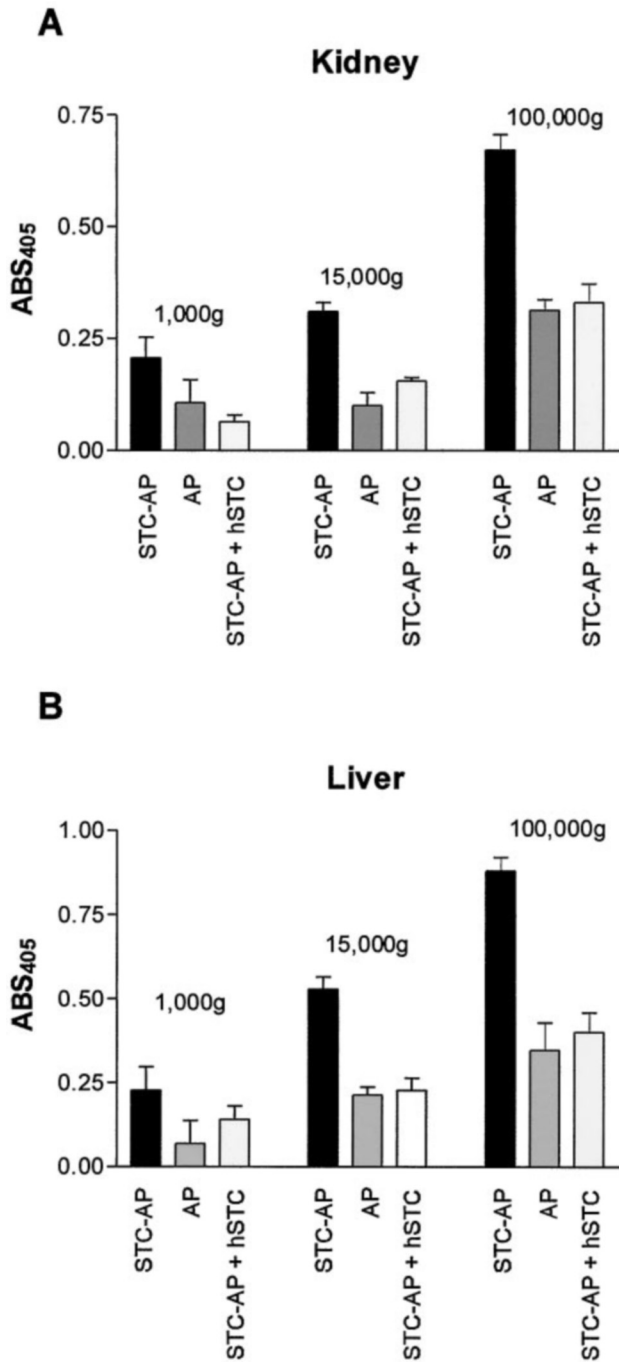


FIG. 2. Binding of STC-AP fusion protein to fractionated rat tissues. Rat kidney and liver tissue was fractionated by differential centrifugation and incubated with 0.5 nM STC-AP, the equivalent amount of AP activity, or 0.5 nM STC-AP plus excess hSTC (for estimation of nonspecific binding). *A*, kidney 1000 \times *g* (nuclei and cell debris), 15,000 \times *g* (microsomes), and 100,000 \times *g* (membranes). *B*, binding to fractionated liver. In both tissues, similar estimates of nonspecific binding were obtained using either non-fusion AP or STC-AP plus 1 μ M recombinant human stanniocalcin ($n = 3$ for each point \pm S.E.). *ABS*, absorbance.

rate based on the disappearance of NADH. For the assay, hSTC (0.5–1000 nM) or a control buffer (50 mM HEPES, pH 7.4) was preincubated with sub-mitochondrial particles for 5 min. The disappearance of NADH in each treatment group was then monitored at 340 nm over the next 15 min to establish NADH consumption rates. Three independent rate determinations were conducted for each concentration of STC.

Statistical Analysis—Saturation binding curves were analyzed using a nonlinear regression analysis of one-site binding in GraphPad Prism where $Y = B_{\max} \times X/(K_d + X)$. For comparison of binding between

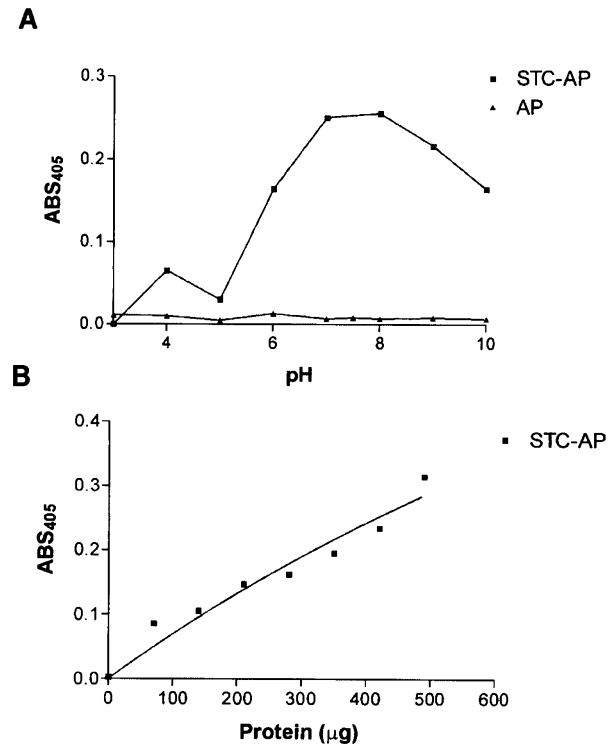


FIG. 3. Binding of STC-AP fusion protein is pH-dependent and linear with protein concentration. *A*, isolated rat liver membranes were incubated with 0.5 nM STC-AP or the equivalent amount of AP activity using acetate (pH 4–6), HEPES (pH 7–8), and Tris (pH 9–10) buffers. Bound AP activity was quantified as described under “Experimental Procedures.” *B*, increasing amounts of rat liver membrane were incubated with fusion protein to establish binding linearity. Graphs are representative of three independent experiments. *ABS*, absorbance.

isolated mitochondria and mitoplasts, a student's *t* test was used ($\alpha = 0.01$). The time course of STC sequestration into intact mitochondria was assessed by one-way ANOVA followed by Dunnett's test using the first time point as the control where $\alpha = 0.05$.

RESULTS

Because radioiodination consistently failed to produce a functional STC tracer for receptor binding assays, we generated a fusion protein (STC-AP) encompassing the entire protein-coding region of the mouse STC cDNA with alkaline phosphatase fused to the C terminus (Fig. 1A). Western blot analysis with STC antiserum revealed that STC-AP was a 210-kDa protein under nonreducing conditions and 120-kDa after reduction with β -mercaptoethanol, suggesting that the STC-AP formed dimers like native STC (Fig. 1B). Identical blots probed with an antibody to placental alkaline phosphatase also revealed the presence of both STC-AP and AP (Fig. 1C). Conditioned media from STC-AP-expressing cells also exhibited parallelism in the RIA, indicating that the fusion protein was immunologically indistinguishable from purified hSTC (data not shown).

We assessed the activity of the AP enzymatic tag and estimated the STC content of conditioned media from STC-AP- and AP-producing cells after concentrating the media using Centriprep devices (Millipore). The specific activity of STC-AP was estimated as 4 milliunits of alkaline phosphatase activity \approx 1 ng of STC (1 unit represents the hydrolysis of 1 mmol of *p*-nitrophenyl phosphate/min at 37 $^{\circ}$ C).

Receptor Binding Assays

Analysis of binding in fractionated kidney and liver tissue revealed some binding in all fractions but highest binding in the 15,000 \times *g* (crude mitochondrial fraction) and 100,000 \times *g*

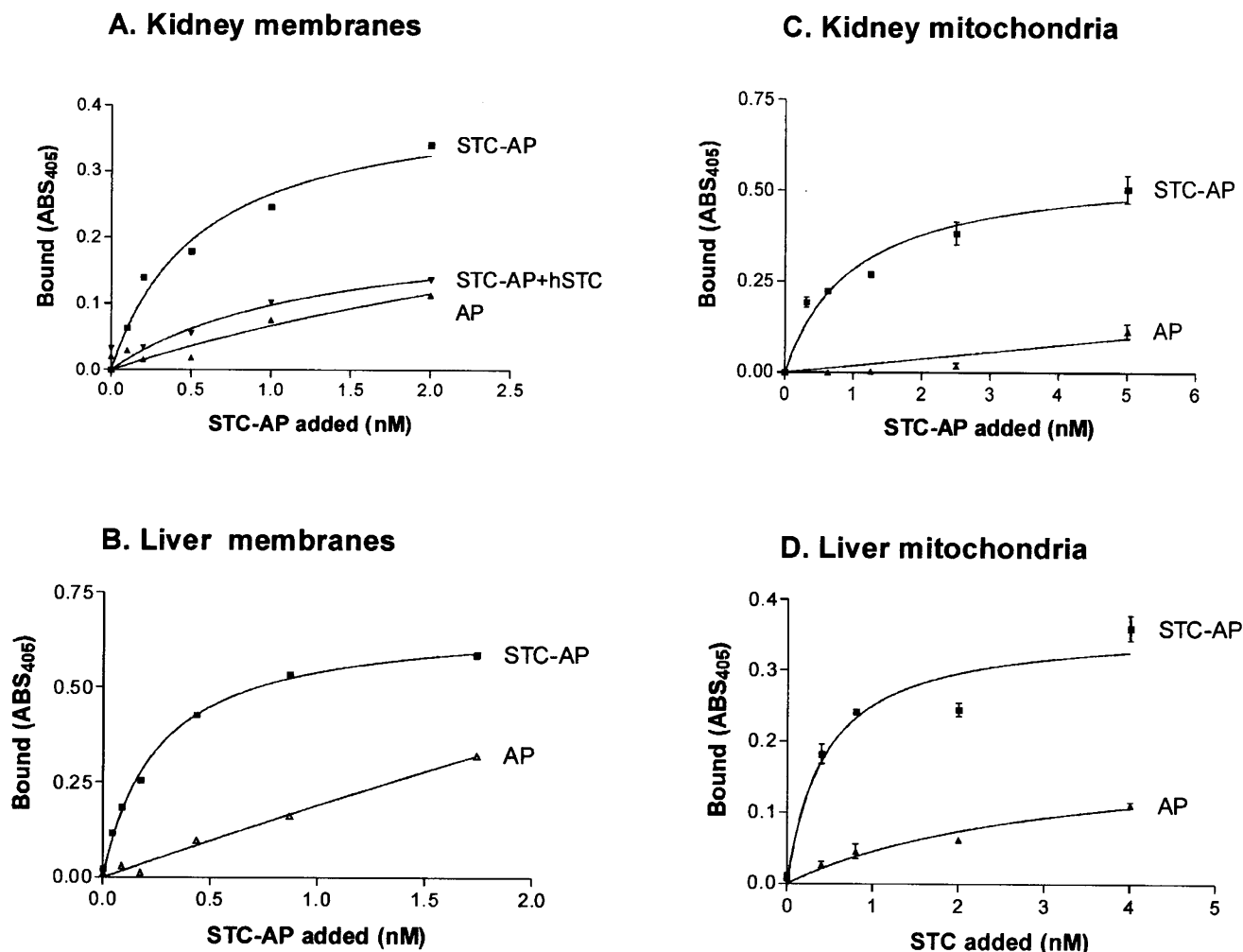


FIG. 4. Saturation binding of STC-AP fusion protein to liver and kidney membranes and mitochondria. Purified kidney and liver membranes and gradient purified mitochondria were incubated with increasing amounts of concentrated conditioned media from MDCK cells expressing STC-AP and AP proteins at room temperature for 90 min. A, analysis of kidney saturation data yielded an affinity of 0.8 nM and a B_{\max} of 1.2 pmol/mg. For estimation of nonspecific binding, 1 μ M hSTC was added to the STC-AP, or AP alone was used. B, analysis of liver saturation binding data yielded a K_d of 0.25 nM and a B_{\max} of 1.8 pmol/mg of protein. C and D, saturation binding analysis of purified kidney (K_d 0.56 nM; B_{\max} 0.99 pmol/mg) and liver (K_d of 0.44; B_{\max} of 1.1 pmol/mg) mitochondria. Membrane saturation curves are representative of six independent determinations. Mitochondrial data are representative of 3 independent determinations (presented as mean \pm S.E.; $n = 3$ per point). ABS, absorbance.

(plasma membranes) fractions (Fig. 2). Nonspecific binding was essentially the same using either AP alone or STC-AP plus 1 μ M hSTC. Using isolated liver plasma membranes, we determined that binding was linear with protein concentration and highly pH-dependent such that binding was reduced outside the physiological range (Fig. 3). Displacement assays using a variety of bioactive NIH hormones and STC-2 demonstrated the specificity of STC-AP binding, because only hSTC was capable of causing significant displacement (not shown).

Purified kidney and liver membranes were analyzed further in saturation binding assays, yielding high affinity, high capacity binding sites in both (Fig. 4, A and B). An estimated K_d of 0.8 ± 0.3 nM and B_{\max} of 1.2 ± 0.6 pmol/mg of protein was obtained for kidney membranes, and a K_d of 0.25 ± 0.1 nM and B_{\max} of 1.8 ± 0.5 pmol/mg of protein was determined for liver membranes (saturation curves are representative of 6 independent determinations).

Because we observed relatively high levels of specific binding to the $15,000 \times g$, mitochondria-rich fraction in liver and kidney, we explored the possibility of STC receptors on mitochondria in greater detail using purified mitochondrial preparations from rat liver and kidney. Saturation binding analysis of

purified mitochondria from rat liver and kidney revealed similar binding kinetics to those obtained with membranes (Fig. 4, C and D, kidney mitochondria had a K_d of 0.56 nM and B_{\max} of 0.99 pmol/mg of protein, and liver mitochondria yielded a K_d of 0.44 nM and B_{\max} of 1.1 pmol/mg of protein. Enzyme analysis revealed that these mitochondria were enriched for succinate dehydrogenase and had minimal contamination by other organelles as compared with a crude homogenate (Fig. 5).

Sub-mitochondrial Localization of STC Receptors

To identify mitochondrial compartments containing the majority of STC receptors, we performed a comparative binding analysis on equal numbers of whole mitochondria and mitoplasts (digitonin-treated mitochondria). Surprisingly, mitoplasts contained twice the number of binding sites as mitochondria (Fig. 6), suggesting that the majority of STC receptors were confined to mitoplast membranes.

Because of the marked differences between mitoplast and mitochondrial binding, we explored the possibility of *in vitro* ligand sequestration by performing a time course study of STC-AP binding to whole mitochondria followed by proteinase K treatment to destroy non-internalized ligand. The results

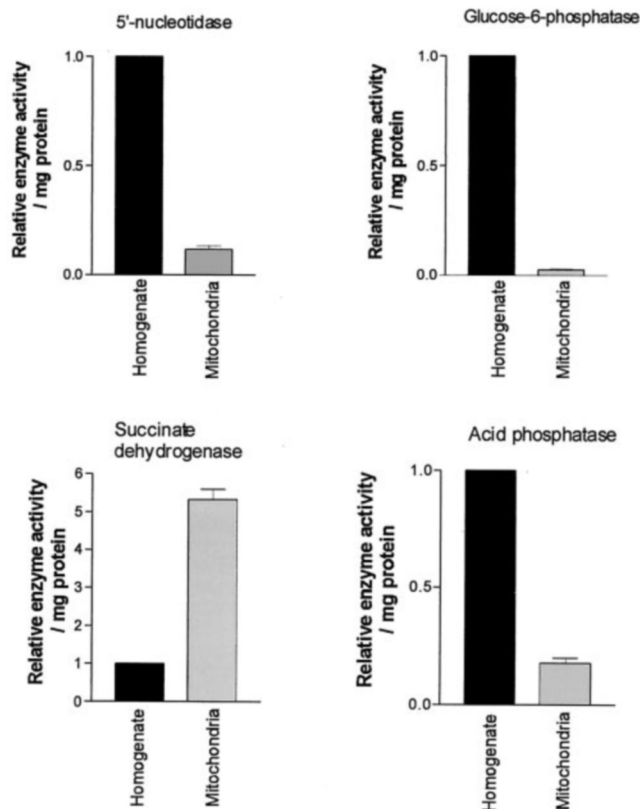


FIG. 5. **Assessment of mitochondrial purity.** Rat liver mitochondria were purified using Percoll gradient centrifugation and subjected to 5'-nucleotidase, acid phosphatase, glucose-6-phosphatase, and succinate dehydrogenase assays to determine the relative levels of plasma membranes, lysosomes, endoplasmic reticulum, and mitochondria (mito.) respectively. Relative enzyme activity was compared with a crude liver homogenate (CH). $n = 3$ for each point \pm S.E.

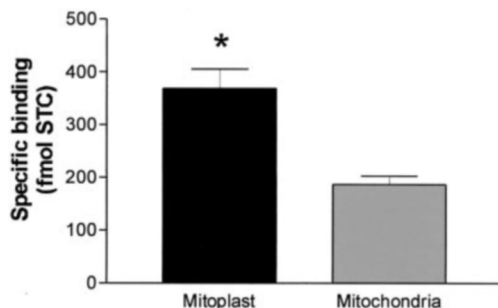


FIG. 6. **Sub-mitochondrial localization of STC receptors.** Mitochondria and mitoplasts were subjected to binding analysis with STC-AP fusion protein and AP for estimation of nonspecific binding. Mitoplasts yielded significantly higher binding than whole mitochondria ($p < 0.01$) indicated by an asterisk. Values represent the mean \pm S.E. where $n = 4$.

demonstrated that there was progressive protection of the ligand from the enzyme over time (Fig 7), suggesting that the native ligand likely undergoes the same process *in vivo*.

Histological Localization of STC Receptors

Kidney—In support of the receptor binding data, *in situ* ligand binding studies with STC-AP revealed discrete, displaceable binding to cells in specific nephron segments. Fig. 8 is a sagittal view of outer medullary kidney in adult mouse after *in situ* ligand binding with STC-AP. The dark brown staining over the cytoplasm of all thick ascending limb cells (asterisks) is representative of specific ligand binding. Specific binding was also observed over collecting duct (CD) cells. This was more

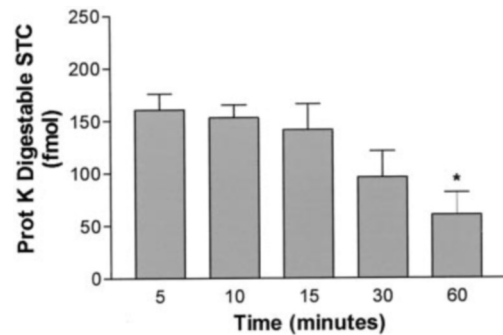


FIG. 7. **STC sequestration by mitochondria.** Intact rat liver mitochondria were incubated with STC-AP fusion ligand for the indicated times followed by digestion with proteinase K (Prot K). The decline over time in proteinase K-digestible STC represents mitochondrial sequestration of the hormone. The asterisk indicates a significant difference from 5- and 10-min time points. Values are presented as means \pm S.E. ($n = 8$).

apparent at higher magnifications, as shown in Fig. 8B, which is a transverse section of outer medullary kidney of another animal. The specific binding to CD and thick limb cells contrasted sharply with the complete absence of binding to the thin loops of Henle (arrows) and vascular bundles (VB) comprising the vasa recta capillaries.

An entirely different pattern of binding was evident in cells comprising the cortical CDs and distal convoluted tubules. Fig. 8C is a view of mouse cortical kidney. The binding of STC-AP to these segments was punctate in appearance and more intense than the binding to medullary CDs or thick limb cells, as evidenced by the darker hue of the reaction product. In these segments, binding was almost entirely restricted to cytoplasmic inclusions that were rod-shaped in appearance and ~ 3 – $5 \mu\text{m}$ in length, highly reminiscent of mitochondria.

Liver—For comparison with kidney, *in situ* ligand binding studies were also carried out on tissue sections of human and mouse liver (Fig. 8, D and E). Here the pattern of binding was the same as that observed in the distal convoluted tubules; hepatocytes demonstrated a high density of punctate, cytoplasmic staining to rod-shaped structures. Binding was also evident on liver cell membranes, particularly in the vicinity of central veins. It is perhaps noteworthy that not all hepatocytes exhibited binding activity.

In control tissue sections, all of the aforementioned ligand binding was displaced by co-incubation of the fusion protein with excess ($1 \mu\text{M}$) recombinant hSTC (Fig. 8, B (inset) and F). Furthermore, as in displacement assays, the addition of $1 \mu\text{M}$ STC-related protein (STCrP or STC2) did not displace fusion protein binding.

STC Localizes to the Inner Mitochondrial Matrix

In view of the presence of STC receptors on mitochondria, we also attempted to localize the STC ligand at the subcellular level using a number of methods. The first entailed a crude subcellular fractionation of kidney and liver tissue followed by RIA quantification of STC in each fraction. This procedure revealed that $>90\%$ of the STC was confined to the 10,000 and $15,000 \times g$ fractions that are largely comprised of heavy and light mitochondria, respectively. The remaining immunoreactivity ($<5\%$) was confined to the $100,000 \times g$ supernatant representing the cytoplasm (Table I). This was also confirmed by Western blot analysis of isolated mitochondrial fractions from liver and kidney. In both instances a single 50-kDa band, approximately the same size as recombinant hSTC, was observed. Pre-absorbing the antiserum with $1 \mu\text{g/ml}$ hSTC eliminated the immuno-detection of this band (not shown).

To further confirm the cell fractionation studies above, im-

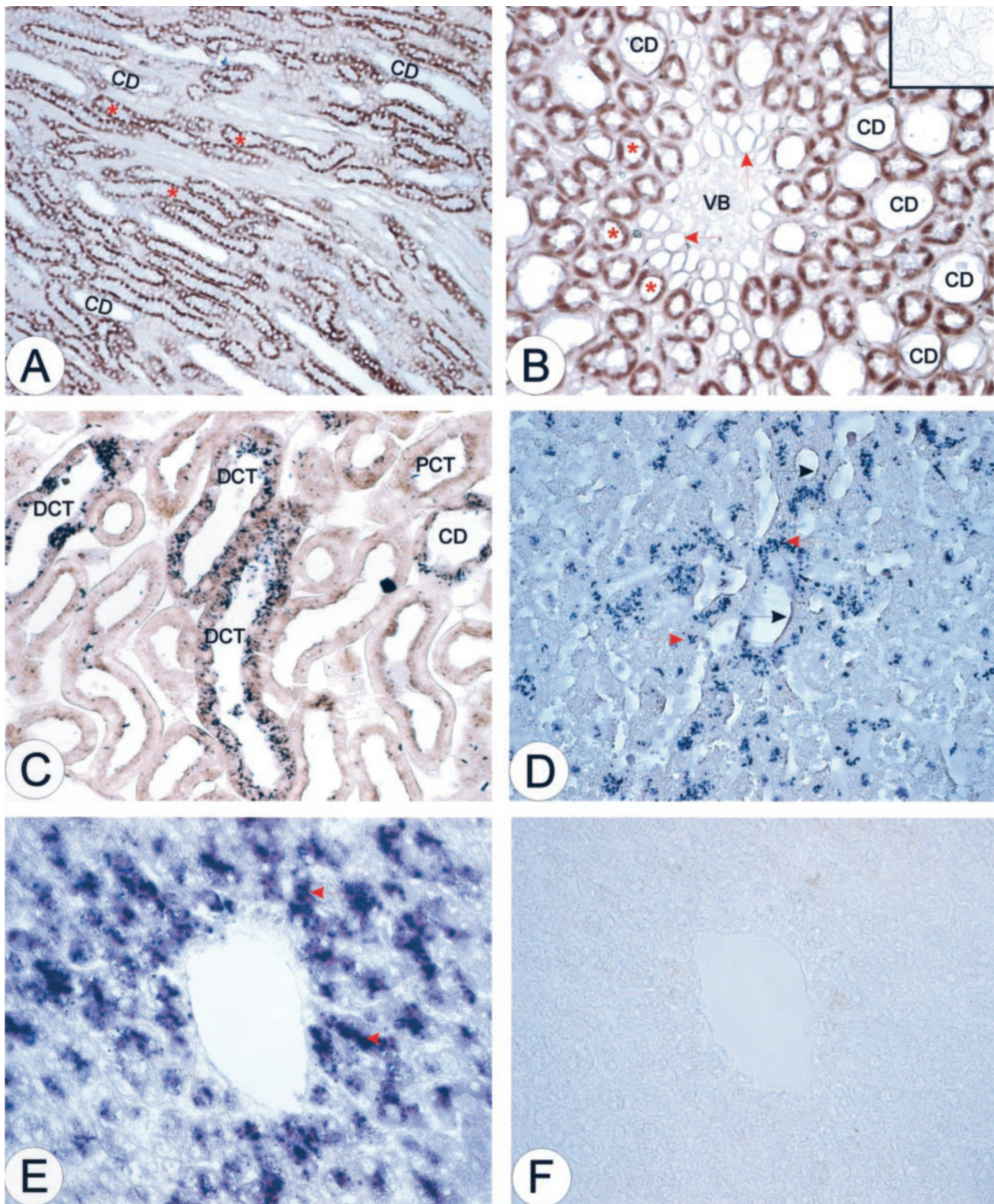


FIG. 8. *In situ* ligand binding of STC-AP fusion protein to kidney and liver sections. *A*, binding of STC-AP to thick ascending limbs (asterisks) and CDs of a sagittal section of mouse outer medullary kidney ($\times 200$). *B*, higher magnification transverse section of outer medullary kidney showing homogeneous cytoplasmic binding in thick limb cells (asterisks) and weaker binding in CDs. No binding was evident in the vascular bundles (VB) and thin loops of Henle (arrows). The inset (upper right) shows a control tissue section ($\times 400$). *C*, mouse outer cortical kidney showing specific binding over distal convoluted tubules (DCTs) and CDs. Apical membrane staining was also evident in proximal convoluted tubules (PCT) ($\times 600$). *D*, rat liver also contained intense cytoplasmic staining (red arrow) and weaker membrane binding (black arrow) ($\times 200$). *E*, human liver biopsy demonstrating punctate staining in most hepatocytes (red arrow) ($\times 600$). *F*, control liver section adjacent to *E* using excess hSTC to compete with the STC-AP fusion protein ($\times 600$).

TABLE I
Subcellular distribution of STC in major organs (ng/mg of total protein)

Tissues were extracted as described under "Experimental Procedures." Equal amounts of protein (0.4–1.0 mg/tube) were then analyzed by RIA. Spleen and lung are included for comparison. ND, not detectable below RIA detection limit of 0.2 ng/ml.

	700 × g nuclear	10,000 × g heavy mitochondria	17,000 × g light mitochondria	100,000 × g membrane	100,000 × g cytosol	% STC in combined mitochondrial fractions
Kidney	ND	18.7	15.2	1.1	1.4	93
Liver	ND	18	15.3	ND	0.78	98
Spleen	ND	16.3	10	ND	1.8	94
Lung	ND	18.1	11	1.5	0.9	92

munogold electron microscopy was performed on adult mouse kidney using STC antiserum. By this procedure, large numbers of colloidal gold particles were preferentially localized over the mitochondria of proximal straight tubules (Fig. 9A), thick ascending limbs (Fig. 9B), distal convoluted tubule (Fig. 9C), and CD cells (Fig. 9D). Colloidal gold staining of the nephron cell cytoplasm was also observed but not nearly to the same extent. All gold labeling was completely abolished in control tissue section by pre-absorbing the antiserum with excess hSTC (Fig. 9A, *inset*), indicating that the procedure was specific for STC.

To pinpoint more precisely the location of mitochondrial STC, the STC content of isolated mitochondria was assessed after treatment with either proteinase K or digitonin. The results showed that proteinase K digestion did not significantly alter the levels of immuno-assayable STC as compared with untreated controls (0.59 ng/mg of protein after digestion as compared with 0.54 ng/mg in controls). This suggested that the majority of STC was inside the mitochondria as opposed to being bound to the outer membrane. However, when 1% digitonin was employed to remove the outer mitochondrial membrane and the intermembrane proteins, the immunoassayable STC content of the remaining mitoplasts remained unchanged as compared with controls (0.61 ng/mg of protein after digitonin as compared with 0.55 ng/mg in untreated controls). This indicated that the majority of STC was confined to the mitoplast. In comparison, control mitochondria that were lysed before proteinase K treatment had STC levels that were below RIA detection limits (<0.2 ng/ml), indicating the effectiveness of proteinase K digestion. The efficacy of digitonin treatment was also assured by Western blot analysis using cytochrome *c* (intermembrane space marker) and COX IV (inner matrix marker) antibodies. Blots revealed that mitoplasts contained very little cytochrome *c* immunoreactivity but similar levels of COX IV as compared with untreated, control mitochondria (data not shown).

STC Effects on Electron Transfer

The presence of STC within the mitoplast was indicative of possible effects on mitochondrial function. To address this, biologically active hSTC (20) was added to sub-mitochondrial particles isolated from bovine heart for assessment of its effects on electron transfer rate (based on the disappearance of NADH). In a series of studies we observed that hSTC had concentration-dependent stimulatory effects on electron transfer that were apparent at concentrations as low as 5 nM and began to plateau at 100 nM (maximum 21.2% and an EC_{50} of 72 nM; Fig. 10). Because the Mitoscan assay system employs sub-mitochondrial particles that are revealed in an "inside out" orientation, the results can be interpreted in at least two ways. If STC was an electron acceptor it could divert electrons away from the respiratory chain, thereby increasing the disappearance of NADH. Alternatively, STC could have direct, stimulatory effects on electron transport through enhancement of respiratory enzyme activity.

DISCUSSION

This report outlines what appears to be a novel signaling and trafficking pathway for the hormone stanniocalcin. It also constitutes the first characterization of saturable and displaceable, high affinity STC receptors on membranes and mitochondria and the first evidence for direct effects of STC on mitochondrial metabolism. As such, STC joins an exclusive list of regulatory factors including nitric oxide (30) and thyroid hormone (31) that have direct effects on electron transport. The only other known polypeptide hormone to be targeted to mitochondria is transforming growth factor $\beta 1$ (3). However, to the best of our knowledge, this is the first reported identification of polypeptide hormone receptors on mitochondria.

Our initial attempt to identify STC receptors relied on 125 I-labeled hSTC as ligand. We are now convinced that radioiodination damages the hormone, rendering it incapable of receptor binding. As an alternative, we created a soluble STC-AP fusion protein for use in receptor binding assays and for localizing STC receptors at the cellular level. First described by Flanagan and Leder (23), this non-isotopic labeling technique has been used to clone a number of receptors such as neuropilin (32) and leptin (33) and ligands such as kit and ELF-1 (34). For our purposes, stable STC-AP transfectants were generated in MDCK cells, whereas a line producing secreted AP was generated as a control. Media harvested from the STC-AP-producing lines contained high levels of a 210-kDa stanniocalcin immunoreactive (STC_{ir}) protein, whereas control cell media only contained high levels of secreted alkaline phosphatase. The STC-AP fusion protein also displayed parallelism in our RIA and, therefore, could be reliably quantified for use in receptor binding studies.

Having established the physical similarities of STC-AP, we proceeded to explore its utility in classical receptor binding studies. Binding assays performed on a variety of fractionated tissues revealed that kidney and liver had the highest number of specific binding sites. Saturation analysis indicated that both contained high affinity binding sites (0.25–0.8 nM) that were completely displaceable with purified hSTC. Because STC is only measurable in serum during pregnancy where blood levels peak at 0.02 nM or 1.5 ng/ml (24), these receptors are in all likelihood insensitive to blood-borne hormone. However, as befitting a molecule that signals locally, estimates of tissue STC content in liver and kidney range from 0.4 to 2.0 ng/kg (24), well within the range of their respective receptor affinities. Notably, several other tissues showed lower levels of specific binding, including lung, brain, skeletal muscle, and spleen. Because all of these latter tissues express the STC gene and/or contain high levels of STC immunoreactivity (35–37), it was not surprising that they exhibited varying degrees of specific binding. The precise localization of receptors in these tissues will form the basis of future studies.

Receptor binding assays on liver and kidney each revealed the presence of STC receptors on mitochondria as well as plasma membranes. *In situ* ligand binding on kidney demon-

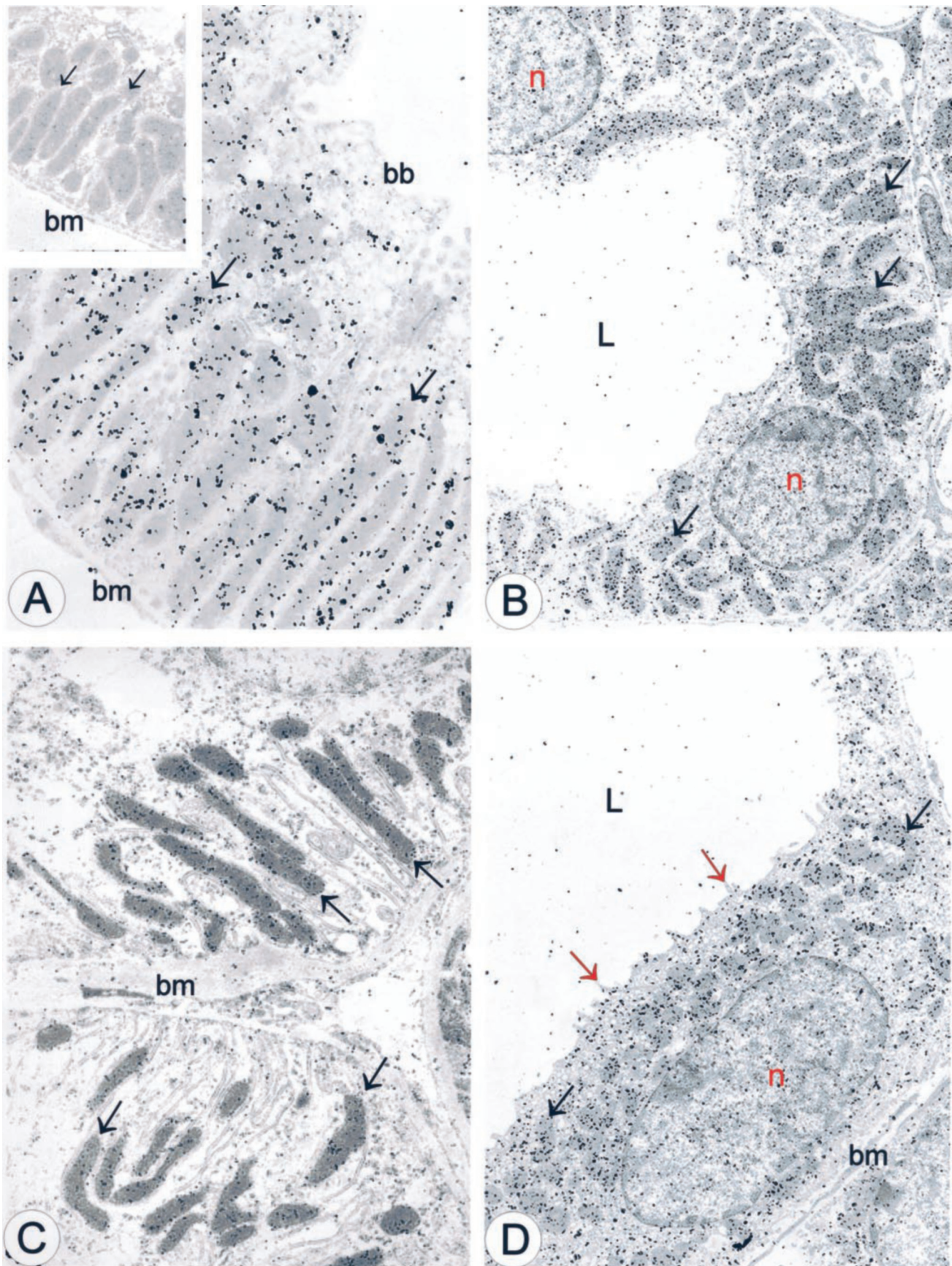


FIG. 9. Immunogold labeling of STC protein in mouse kidney mitochondria. Note dense labeling (black deposits) over mitochondria in proximal straight tubule (arrows in A), thick ascending limbs (B), distal convoluted tubules (C) and a cortical collecting duct α -intercalated cell (D). The inset in panel A shows an absence of staining when antiserum was preabsorbed with hSTC (control). L, lumen; bb, brush border; bm, basement membrane; n, nucleus.

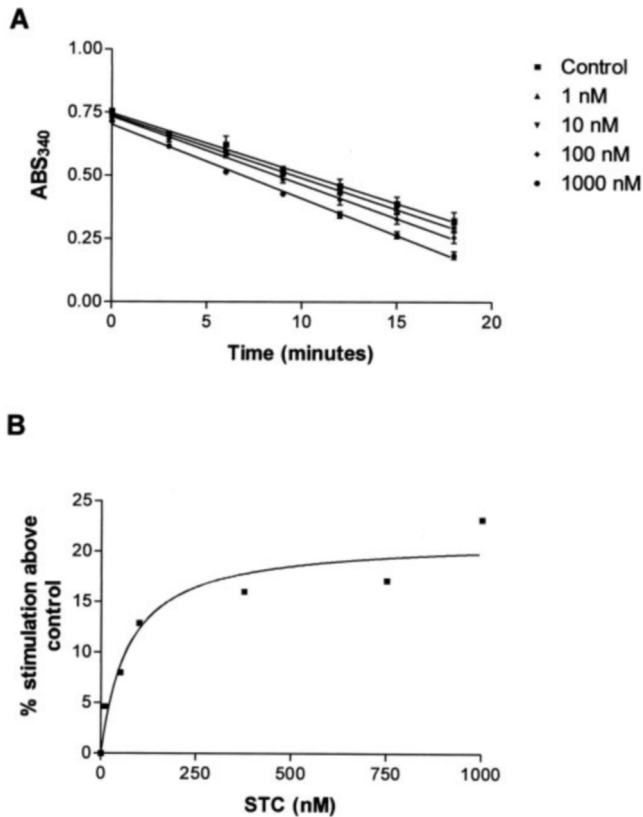


FIG. 10. Stanniocalcin stimulates electron transfer in sub-mitochondrial particles. A, beef heart sub-mitochondrial particles were incubated with human recombinant STC or control buffer, and the rate of NADH was determined at 340 nm. B, STC stimulated electron transport at a maximal level of 21.2% with an EC_{50} of 72.3 nM. ABS, absorbance.

strated punctate staining in cells comprising the distal convoluted tubules that are known to have the highest mitochondrial density in the nephron (38). Based on the binding and uptake studies with purified mitochondria and the size and shape of the subcellular structures revealed by *in situ* ligand binding, we believe that the intense punctate staining pattern observed in kidney and liver cytoplasm is representative of discrete mitochondrial binding. In kidney, only distal convoluted tubule and collecting duct cells exhibited this intense mitochondrial binding. In liver, on the other hand, the mitochondrial binding was the norm but was only evident in a subset of hepatocytes, the significance of which needs to be established.

Although membrane and mitochondrial receptors had similar affinities, it remains to be seen if they are the same. In terms of targeting models, one possible scenario envisions the membrane receptor acting first as a signal transducer and secondly as a chaperone to facilitate translocation and mitochondrial sequestration of the ligand. Alternatively, the membrane-bound receptor may serve solely in the capacity of a signal transducer, which then passes the ligand to a unique mitochondrial receptor for sequestration. It also remains to be seen if the STC receptor is in fact coupled to a signal transduction mechanism. Previous studies suggest that in its actions on fish kidney, STC operates via the protein kinase A pathway by increasing cellular levels of cAMP (39). However, our attempts to replicate these findings in mammalian kidney with hSTC have thus far been unsuccessful.

Although STC receptors in liver were characteristically mitochondrial, or membrane-bound, this was not always the case in kidney. Indeed, the location of renal receptors varied widely according to nephron segment. In distal convoluted tubule and

cortical CD cells they were mostly mitochondrial, as opposed to being sited on the basolateral membranes in medullary CD cells. In thick ascending limbs on the other hand, the positioning appeared exclusively cytoplasmic. The most likely explanation in this case, where the nephron is highly heterogeneous in cellular make-up, is simply that the receptor is being seen in different contextual settings, cytoplasm (trafficking), endoplasmic reticulum (synthesis), membranes/mitochondria (binding), rather than there being fundamental differences in receptor biology along the nephron. Hence, what appears as purely cytoplasmic staining in thick ascending limbs is probably some combination of binding, trafficking, and synthesis. Interestingly, ovarian corpus luteal cells also contain high levels of cytoplasmic receptors for prolactin (40), growth hormone (41), and human chorionic gonadotropin (42).

The discovery of mitochondrial STC receptors prompted a similar search for the ligand, revealing remarkably high levels of STC immunoreactivity in crude kidney and liver mitochondrial fractions. Cell fractionation studies showed that more than 90% of the STC was confined to the heavy and light mitochondrial fractions. In contrast, there was little or no STC present in the cytoplasmic fraction (post 100,000 $\times g$) and very little in the nuclear fraction. The absence of nuclear STC is in marked contrast to parathyroid-related peptide, another broadly distributed calciotropic hormone (1, 2). In support of these cell fractionation studies, electron microscope immunogold labeling demonstrated heavy labeling over mitochondria of all receptor-bearing nephron segments in mouse kidney. Western blots of purified mitochondria revealed that mitochondrial STC was 50 kDa, approximately the same size as human recombinant STC (20, 24).

Because proteinase K and digitonin treatments had no effects on mitochondrial STC content, we concluded that most of the hormone was confined to the inner matrix. More significantly, clues as to its function were deduced in bioassays employing sub-mitochondrial particles. Here we observed that STC had concentration-dependent stimulatory effects on electron transfer to a maximum level of 21%. These findings suggest that STC is capable of accelerating mitochondrial metabolism, perhaps for the enhancement of ATP synthesis. This notion is also supported by recent studies employing mouse transgenesis. Two reports have shown that STC-overexpressing mice are significantly smaller (30–40%) than wild type litter mates but at the same time have enhanced rates of both food and oxygen consumption (22, 47). Interestingly, the degree of enhancement in oxygen consumption (14%) agrees well with our B_{max} for accelerated electron transfer (21%). Such a role for STC might explain its penchant for being targeted to cells with high metabolic activity. Intriguingly, mitochondrial swelling is also seen in tissues with high transgene expression (47). The present data suggest that this was probably due to STC hyperstimulation. Because mitochondrial targeting appears operative in many different tissues, it could be the basis of a unifying hypothesis of hormone action linking STC to the regulation of cellular metabolism. Moreover, it may not be unique to mammals because mitochondrial STC immunoreactivity has recently also been reported in specific nephron segments within the fish kidney, a designated target of STC in lower vertebrates (43).

Several years ago we formulated a sequestering hypothesis of STC action. It arose from the marked discrepancies we observed in the cellular patterns of STC mRNA and protein localization, first in adult kidney, then in adult ovary, and subsequently in most organs of the developing mouse embryo (11, 17, 37, 44–46). In all tissues we observed that STC gene expression was confined to specific cells. However, correlative

immunocytochemistry revealed equally high levels of STC in adjacent cells that had no evidence of STC gene expression. On this basis we formulated a model of STC signaling whereby STC was produced by one cell type and then targeted to and sequestered by adjacent cells. The results of the present study now suggest this model is valid, at least with respect to liver and kidney, by revealing the existence of high affinity receptors on cells previously shown to contain high levels of STC but no evidence of gene expression (11, 46). It is presumably by way of these receptors that STC is sequestered by various cells. Interestingly, cortical and medullary CD cells, which are the principle sites of STC production in rodent kidney, also contained mitochondrial STC and STC receptors, implying that here the hormone is operating in an autocrine feedback loop to regulate CD cell function. The sequestering of STC therefore appears to be a receptor-mediated process, first with in its initial entry to the cell and then possibly in its subsequent shuttling to the mitochondria. Last, stanniocalcin-related protein (STC-2) did not displace the binding of STC-AP to isolated membranes or tissue sections, suggesting that it likely interacts with a different receptor. It remains to be seen if STC-2 also undergoes similar cellular sequestering and targeting to mitochondria.

In summary, we have made significant advances in our understanding of STC signaling and function. We have identified a class of high affinity STC receptors in nephron epithelial cells and liver hepatocytes, present on both the plasma membrane and mitochondria. More significantly perhaps, we have identified STC as a putative regulator of mitochondrial metabolism. The precise nature and underlying purpose of its role in mitochondrial function should be revealed in future studies.

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REFERENCES

- Henderson, J. E., Amizuka, N., Warshawsky, H., Biasotto, D., Lanske, B. M., Goltzman, D., and Karaplis, A. C. (1995) *Mol. Cell. Biol.* **15**, 4064–4075
- Massfelder, T., Dann, P., Wu, T. L., Vasavada, R., Helwig, J. J., and Stewart, A. F. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 13630–13635
- Chen, W., Jin, W., Tian, H., Sicurello, P., Frank, M., Orenstein, J. M., and Wahl, S. M. (2001) *J. Exp. Med.* **194**, 439–453
- Chang, A. C., Janosi, J., Hulsbeek, M., de Jong, D., Jeffrey, K. J., Noble, J. R., and Reddel, R. R. (1995) *Mol. Cell. Endocrinol.* **112**, 241–247
- McCudden, C. R., Kogon, M. R., DiMattia, G. E., and Wagner, G. F. (2001) *J. Endocrinol.* **171**, 33–44
- Olsen, H. S., Cepeda, M. A., Zhang, Q. Q., Rosen, C. A., and Vozzolo, B. L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 1792–1796
- Wagner, G. F. (1994) in *Fish Physiology* (Sherwood, N. M., and Hew, C. L., eds) Vol. 13, pp. 273–302, Academic Press, Inc., New York
- McCudden, C. R., Tam, W. H., and Wagner, G. F. (2001) *Biol. Reprod.* **65**, 763–770
- Wagner, G. F., Vozzolo, B. L., Jaworski, E., Haddad, M., Kline, R. L., Olsen, H. S., Rosen, C. A., Davidson, M. B., and Renfro, J. L. (1997) *J. Bone Miner. Res.* **12**, 165–171
- Madsen, K. L., Tavernini, M. M., Yachimec, C., Mendrick, D. L., Alfonso, P. J., Buerger, M., Olsen, H. S., Antonaccio, M. J., Thomson, A. B., and Fedorak, R. N. (1998) *Am. J. Physiol.* **274**, G96–G102
- Deol, H. K., Varghese, R., Wagner, G. F., and Dimattia, G. E. (2000) *Endocrinology* **141**, 3412–3421
- Lal, A., Peters, H., St Croix, B., Haroon, Z. A., Dewhirst, M. W., Strausberg, R. L., Kaanders, J. H., van der Kogel, A. J., and Riggins, G. J. (2001) *J. Natl. Cancer Inst.* **93**, 1337–1343
- Miura, W., Mizunashi, K., Kimura, N., Koide, Y., Noshiro, T., Miura, Y., Furukawa, Y., and Nagura, H. (2000) *Apmis* **108**, 367–372
- Fujiwara, Y., Sugita, Y., Nakamori, S., Miyamoto, A., Shiozaki, K., Nagano, H., Sakon, M., and Monden, M. (2000) *Int. J. Oncol.* **16**, 799–804
- Nelson, A. E., Namkung, H. J., Patava, J., Wilkinson, M. R., Chang, A. C., Reddel, R. R., Robinson, B. G., and Mason, R. S. (1996) *Mol. Cell. Endocrinol.* **124**, 17–23
- Kahn, J., Mehraban, F., Ingle, G., Xin, X., Bryant, J. E., Vehar, G., Schoenfeld, J., Grimaldi, C. J., Peale, F., Draksharapu, A., Lewin, D. A., and Gerritsen, M. E. (2000) *Am. J. Pathol.* **156**, 1887–1900
- Stasko, S. E., DiMattia, G. E., and Wagner, G. F. (2001) *Mol. Cell. Endocrinol.* **174**, 145–149
- Zhang, K., Lindsberg, P. J., Tatlisumak, T., Kaste, M., Olsen, H. S., and Andersson, L. C. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 3637–3642
- Wong, C. K., Yeung, H. Y., Mak, N. K., DiMattia, G. E., Chan, D. K., and Wagner, G. F. (2002) *J. Endocrinol.* **173**, 199–209
- Zhang, J., Alfonso, P., Thotakura, N. R., Su, J., Buerger, M., Parmelee, D., Collins, A. W., Oelkelt, M., Gaffney, S., Gentz, S., Radman, D. P., Wagner, G. F., and Gentz, R. (1998) *Protein Expression Purif.* **12**, 390–398
- Sheikh-Hamad, D., Rouse, D., and Yang, Y. (2000) *Am. J. Physiol. Renal Physiol.* **278**, 417–424
- Varghese, R., Gagliardi, A. D., Bialek, P. E., Yee, S. P., Wagner, G. F., and Dimattia, G. E. (2002) *Endocrinology* **143**, 868–876
- Flanagan, J. G., and Leder, P. (1990) *Cell* **63**, 185–194
- De Niu, P., Radman, D. P., Jaworski, E. M., Deol, H., Gentz, R., Su, J., Olsen, H. S., and Wagner, G. F. (2000) *Mol. Cell. Endocrinol.* **162**, 131–144
- Graham, J. M., and Higgins, J. A. (1993) *Methods in Molecular Biology*, pp. 19 and 27, Humana Press Inc., Totowa, NJ
- Reinhart, P. H., Taylor, W. M., and Bygrave, F. L. (1982) *Biochem. J.* **204**, 731–735
- Morré, J. D. (1971) in *Methods in Enzymology* (Jakoby, W. B., ed) p. 648, Academic Press, Inc., New York
- Flanagan, J. G., and Cheng, H. J. (2000) *Methods Enzymol.* **327**, 198–210
- Clausmeyer, S., Sturzebecher, R., and Peters, J. (1999) *Circ. Res.* **84**, 337–344
- Cadenas, E., Poderoso, J. J., Antunes, F., and Boveris, A. (2001) *Free Radic. Res.* **33**, 747–756
- Wrutniak-Cabello, C., Casas, F., and Cabello, G. (2001) *J. Mol. Endocrinol.* **26**, 67–77
- He, Z., and Tessier-Lavigne, M. (1997) *Cell* **90**, 739–751
- Tartaglia, L. A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G. J., Campfield, L. A., Clark, F. T., and Deeds, J. (1995) *Cell* **83**, 1263–1271
- Cheng, H. J., and Flanagan, J. G. (1994) *Cell* **79**, 157–168
- Chang, A. C., Dunham, M. A., Jeffrey, K. J., and Reddel, R. R. (1996) *Mol. Cell. Endocrinol.* **124**, 185–187
- De Niu, P., Olsen, H. S., Gentz, R., and Wagner, G. F. (1998) *Mol. Cell. Endocrinol.* **137**, 155–159
- Varghese, R., Wong, C. K., Deol, H., Wagner, G. F., and DiMattia, G. E. (1998) *Endocrinology* **139**, 4714–4725
- Kriz, W., and Kaissling, B. (1992) in *The Kidney: Physiology and Pathophysiology* (Seldin, D. W., and Giebisch, G. H., eds) Vol. 3, 2nd Ed., pp. 707–779, Raven Press, Ltd., New York
- Lu, M., Wagner, G. F., and Renfro, J. L. (1994) *Am. J. Physiol.* **267**, 1356–1362
- Dunaif, A. E., Zimmerman, E. A., Friesen, H. G., and Frantz, A. G. (1982) *Endocrinology* **110**, 1465–1471
- Bergeron, J. J., Posner, B. I., Josefsberg, Z., and Sikstrom, R. (1978) *J. Biol. Chem.* **253**, 4058–4066
- Rao, C. V., and Saxena, B. B. (1973) *Biochim. Biophys. Acta* **313**, 372–389
- Amemiya, Y., Marra, L. E., Reyhani, N., and Youson, J. H. (2002) *Mol. Cell. Endocrinol.* **188**, 141–150
- Haddad, M., Roder, S., Olsen, H. S., and Wagner, G. F. (1996) *Endocrinology* **137**, 2113–2117
- Stasko, S. E., and Wagner, G. F. (2001) *Dev. Dyn.* **220**, 49–59
- Wong, C. K., Ho, M. A., and Wagner, G. F. (1998) *J. Endocrinol.* **158**, 183–189
- Filvaroff, E. H., Guillet, S., Zlot, C., Bao, M., Ingle, G., Steinmetz, H., Hoeffel, J., Bunting, S., Ross, J., Carano, R. A., Powell-Braxton, L., Wagner, G. F., Eckert, R., Gerritsen, M. E., and French, D. M. (2002) *Endocrinology* **143**, 3681–3690