Cloning and characterization of cDNAs encoding steroidogenic acute regulatory protein from freshwater stingrays (*Potamotrygon* spp.)

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Abstract

The steroidogenic acute regulatory protein (StAR) is critical to the regulated synthesis of steroids in vertebrates. We have isolated cDNA sequences encoding StAR in the freshwater stingrays *Potamotrygon hystrix* and *P. motoro*. A single *P. hystrix* StAR transcript (3376 bp) and two overlapping *P. motoro* StAR transcripts (1272 and 3365 bp) were isolated. The *P. hystrix* and *P. motoro* StAR transcripts contain open reading frames encoding proteins of 284 amino acids that are 99% identical to each other and 56–64% identical to other StAR proteins. Pregnenolone synthesis by green monkey kidney (COS-1) cells transfected with an expression construct encoding a human cholesterol side chain cleavage/adrenodoxin reductase/adrenodoxin fusion protein was increased 16-fold by coexpression with a pCMV5/*P. motoro* StAR expression construct. Northern blot analysis revealed a single 4000 bp StAR transcript in the *P. motoro* interrenal gland, but RT-PCR indicates StAR mRNA is also expressed in the brain, gonads, atria, ventricle, gill (female only) and muscle (female only). Expression in extragonadal and extraadrenocortical tissues is an indication that StAR may be critical to processes other than steroidogenesis. The longest *P. motoro* StAR transcript contains a sequence with great similarity to short interspersed repetitive elements found in other elasmobranchs. This study is the first to isolate and characterize elasmobranch StAR cDNA sequences and to demonstrate the activity of a nonmammalian StAR protein in a heterologous expression system.

Journal of Molecular Endocrinology (2005) **35**, 557–569

Introduction

Steroid hormones cannot be stored intracellularly and must be synthesized on demand. The single most important factor regulating the timing and rate of steroidogenesis appears to be the steroidogenic acute regulatory protein (StAR) (for review see Stocco 2001). StAR belongs to a family of proteins that share a cholesterol-binding motif termed the StAR-related lipid transfer (START) domain; this domain accommodates cholesterol and/or cholesterol-like sterols (Strauss *et al.* 2003). The START family is diverse, including StAR-like MLN-64 proteins and phosphatidylcholine transfer proteins from animals and Glabra2 homeobox proteins from plants, among other proteins (Strauss *et al.* 2003).

StAR proteins facilitate the transfer of cholesterol, the precursor of all steroids, from the outer mitochondrial membrane to the inner mitochondrial membrane, where cholesterol is converted into pregnenolone by cytochrome P450 cholesterol side chain cleavage enzyme (SCC) (reviewed by Stocco 2001). As the synthesis of a diverse group of steroids, each having distinctive

physiological effects, depends on the conversion of cholesterol to pregnenolone, StAR-mediated steroidogenesis plays an important role in several biological processes. For example, the timely synthesis of steroids is crucial for successful reproduction (e.g. sex steroid synthesis), maintaining ionic homeostasis (e.g. mineralocorticoid synthesis) and for responding to stress (e.g. glucocorticoid synthesis).

Identification of the factors that regulate steroidogenesis can help define the physiological role of specific steroids, because steroids cannot be sequestered and therefore must be made when needed. For example, adrenocorticotrophic hormone (ACTH) is released by the pituitary in response to stress and induces glucocorticoid synthesis. Similarly, angiotensin II (AII) is involved in the regulation of salt and water balance and mediates its actions in part by inducing the synthesis of mineralocorticoids. In mammals, factors such as ACTH and AII are known to induce steroidogenesis through a process that is sensitive to inhibitors of translation (e.g. cycloheximide), but insensitive to inhibitors of transcription (e.g. actinomycin D) (Ferguson 1962, 1963, Garren *et al.* 1965, 1966). Recent work indicates that the

cycloheximide-sensitive induction of steroidogenesis is dependent on the rapid translation of StAR mRNA (for review see Stocco 2001).

In fishes, a single steroid is thought to serve as both a mineralocorticoid and a glucocorticoid, but the precise role of corticosteroids in these animals is poorly defined. Characterization of the structure and regulation of StAR from fishes will greatly enhance our understanding of the role of steroidogenesis in maintaining homeostasis in these animals. The sequences of cDNA clones encoding teleost forms of StAR have recently been determined and studies of StAR regulation in gonadal and adrenocortical tissues of teleosts have been conducted (Bauer *et al.* 2000, Kusakabe *et al.* 2002b, von Hofsten *et al.* 2002, Li *et al.* 2003, Geslin & Auperin 2004). However, the nature of the teleost head kidney, a tissue composed of adrenocortical as well as several other cell types, complicates the study of steroidogenesis in these fishes. The elasmobranch interrenal gland is a good model tissue to study StAR in fishes as it is a discrete, encapsulated gland composed of a single cell type whose sole function is to produce the corticosteroid 1 α ,11 β ,21-trihydroxy-4-pregnene-3,20-dione (1 α -hydroxycorticosterone; 1 α -B) (Idler & Truscott 1966, Idler *et al.* 1967).

To date, no study has directly examined the regulation and role of StAR in elasmobranch steroidogenesis. Previous work has shown that (i) the rate of interrenal gland steroidogenesis in marine elasmobranchs is increased by ACTH (Klesch & Sage 1973, Hazon & Henderson 1985, Armour *et al.* 1993, Nunez & Trant 1999); (ii) the induction of steroidogenesis by ACTH in the Atlantic stingray, *Dasyatis sabina* is sensitive to cycloheximide, but not actinomycin D (Nunez & Trant 1999); and (iii) AII induces 1 α -B synthesis in the interrenal glands of the narrowly euryhaline European lesser spotted dogfish (*Scyliorhinus canicula*) (Hazon & Henderson 1985, Armour *et al.* 1993, Anderson *et al.* 2001), but not in the fully euryhaline *D. sabina* (Nunez & Trant 1999). These investigations indicate that the regulation of adrenocortical steroidogenesis may differ between elasmobranch species with different salinity tolerances. Therefore, in order to define the homeostatic role of corticosteroids in elasmobranchs, it will be necessary to examine the regulation of interrenal gland steroidogenesis in stenohaline and euryhaline species.

Stingrays of the family Potamotrygonidae are stenohaline freshwater elasmobranchs that cannot survive in seawater because of their inability to retain nitrogenous osmolytes (i.e. urea and trimethylamine oxide) and reduced size of their salt-secreting rectal gland compared with marine elasmobranchs (Thorson *et al.* 1967, 1978, Thorson 1970, Gerst & Thorson 1977). Because they obviously face very different ionoregulatory problems than marine elasmobranchs, Potamotrygonid rays are excellent comparative models relative to euryhaline species such as *D. sabina*. Indeed, there is a

growing body of literature regarding ionoregulation in these animals (for example, see Thorson 1970, Carrier & Evans 1973, Gerst & Thorson 1977, Wood *et al.* 2002, 2003). The goals of the current investigation were to isolate and characterize cDNA clones encoding StAR from the interrenal glands of freshwater stingrays (*Potamotrygon hystrix* and *P. motoro*) because of the importance of StAR to the regulated synthesis of corticosteroids that are involved in elasmobranch ionoregulation and other homeostatic processes (e.g. the stress response).

Materials and methods

Animals

Initial studies were conducted using tissues collected from *P. hystrix*, which were purchased from International Fisheries (Miami, FL, USA) and held in accordance to guidelines established by the University of Florida's Institutional Animal Care and Use Committee. When it became difficult to obtain verified specimens of *P. hystrix* from commercial sources, *P. motoro* (a closely related species) were purchased from The Aquarium of the Americas (New Orleans, LA, USA). The Aquarium of the Americas has maintained a breeding colony of captive *P. motoro* for 13 years and employs experts in the husbandry and biology of freshwater stingrays. Upon arrival at the University of Texas Marine Science Institute, the animals were anesthetized in 0.05% MS-222 and pithed by spinal transection.

Isolation of freshwater stingray StAR cDNA sequences

Tissues were excised immediately from the pithed stingrays, then frozen in liquid nitrogen and stored at -80°C until use. Total RNA was isolated with Tri-Reagent (Sigma) following the manufacturer's instructions and used as template for reverse transcription (RT) (Clontech). In standard RT reactions, total RNA (1 μg) mixed with 100 ng random hexamer primers (10 μl total volume) was heated at 70°C for 10 min and then chilled on ice for 2 min. First-strand reaction buffer (1 $\times$ final concentration), dithiothreitol (10 mM final concentration), dNTPs (1 mM final concentration), RNase inhibitor (40 units; Invitrogen) and Powerscript reverse transcriptase (1 μl enzyme mix; Clontech) were added and the reactions incubated for 10 min at 25°C . Reactions were then incubated at 42°C for 1 h and terminated by heating at 70°C for 10 min. RT reactions were subsequently diluted 1:10 with DNase-free water and used as template for the PCR.

P. hystrix interrenal gland RNA was initially used as template for RT-PCR to obtain cDNA clones encoding freshwater stingray StAR. One-tenth of a *P. hystrix*

interrenal gland RT reaction was added to a total volume of 25 µl containing 1 × Advantage 2 reaction buffer (Clontech), 1 mM dNTPs, 20 pmol each sense (5'-GCATGGARGCNATGGGNGAGTGGAA-3') and antisense (5'-TYTTNGGCAGCCANCCYTTSAG GTC-3') primers (as described in Bauer *et al.* 2000) and 0.5 µl Advantage 2 Polymerase mix (Clontech). PCR thermal parameters were: 95 °C for 2 min followed by 35 cycles of 30 s at 95 °C, 30 s at 45 °C and 30 s at 72 °C. Products of this reaction were resolved on a 2% agarose gel in Tris–borate (90 mM), EDTA (2 mM) buffer (TBE) and visualized with ethidium bromide. Amplicons of the appropriate size were ligated into the pCRII vector (Invitrogen) and used to transform competent *Escherichia coli* (Top 10; Invitrogen). Plasmid DNA was isolated from bacteria using a commercial plasmid isolation procedure (Sigma) and sequenced to determine the identity of inserted DNA.

Rapid amplification of cDNA ends (RACE) was used to isolate the 5'- and 3'-ends of freshwater stingray StAR mRNAs. RACE-ready cDNA libraries were synthesized using *P. hystrix* interrenal gland total RNA and a commercial kit (SMART RACE; Clontech), following the manufacturer's protocol. Both 5'-RACE and 3'-RACE first-strand reactions were diluted 1:10 with Tricine–EDTA (10 mM and 1 mM respectively). To obtain the 3'-end of *P. hystrix* StAR transcripts, one-tenth of the 3'-RACE first-strand reaction was added to a total volume of 25 µl containing 1 × Advantage 2 reaction buffer, 1 mM dNTPs, 20 pmol *P. hystrix* StAR-specific sense primer (5'-CTGCAGAACTGCGGGCAATGT GATCAG-3'), 2.5 µl 10 × SMART RACE universal primer mix and 0.5 µl Advantage 2 polymerase mix. The Advantage 2 polymerase mix contains a small amount of proofreading polymerase and has three times the fidelity of standard thermostable polymerase mixes. The 5'-end of the *P. hystrix* StAR transcript was obtained in a similar manner except one-tenth of the 5'-RACE first-strand reaction was used as template and 20 pmol *P. hystrix* StAR-specific antisense primer (5'-GGCCAT CCCTGCTAGGAAGCAAGTTGAG-3') were used as the gene-specific primer. Thermal parameters for RACE touchdown PCR were: 95 °C for 2 min; five cycles of 95 °C for 5 s and 72 °C for 180 s; five cycles of 95 °C for 5 s, 70 °C for 10 s and 72 °C for 180 s; 25 cycles of 95 °C for 5 s, 68 °C for 10 s and 72 °C for 180 s. Products of these reactions were resolved on a 1% agarose gel in TBE and visualized with ethidium bromide. PCR products were ligated into the pCR II cloning vector (Invitrogen) and sequenced. To obtain StAR sequence from *P. motoro*, we synthesized RACE-ready cDNA libraries from *P. motoro* interrenal gland total RNA as described above. *P. hystrix* and *P. motoro* StAR primers were then used in standard RT-PCR, 5'-RACE and 3'-RACE reactions to obtain *P. motoro* StAR sequence. To obtain accurate consensus sequences of the *P. hystrix*

and *P. motoro* StAR mRNAs, at least three different 5'-RACE and 3'-RACE clones were sequenced in both directions. The open reading frames encoding *P. hystrix* and *P. motoro* StAR were sequenced at least three times more in both directions during the creation of expression constructs.

Sequence alignments and molecular phylogenetic analysis

To determine their relationship to other members of the START family, the deduced amino acid sequences of the putative *P. hystrix* and *P. motoro* StAR proteins were included in molecular phylogenetic analysis with the sequences of *Salvelinus fontinalis* (brook trout; accession AAG39689), *Oncorhynchus mykiss* (rainbow trout; accession BAB18779), *Anguilla japonica* (Japanese eel; accession BAC66210), *Danio rerio* (zebrafish; accession AAH75967), *Gadus morhua* (Atlantic cod; accession AY291434.1), *Xenopus laevis* (African clawed frog; accession Q9DG08), *Gallus gallus* (chicken; accession Q9DG09), *Taeniopygia guttata* (zebrafinch; accession AY505123.1), *Mus musculus* (mouse; accession P51557), *Mesocricetus auratus* (hamster; accession P70114), *Rattus norvegicus* (rat; accession P97826), *Equus caballus* (horse; accession O46689), *Sus scrofa* (pig; accession Q28996), *Ovis aries* (sheep; accession P79245), *Bos taurus* (cow; accession Q28918) and *Homo sapiens* (human; accession P49675) StAR proteins, *Caenorhabditis elegans* (accession NP_498027), zebrafish (accession AAG28603; partial amino acid sequence), *Xenopus tropicalis* (accession AAH76666), mouse (accession BC003313) and human (accession CAA56489) MLN64, mouse (accession Q99JV5) and human (accession Q96DR4) START-domain-containing 4 protein (STARD4), mouse (accession Q9 EPQ7) and human (accession Q9 NSY2) STARD5, mouse (accession P59096) and human (accession P59095) STARD6, mouse (accession Q9 JMD3) and human (accession Q9Y365) STARD10, mouse (accession Q923Q2) and human (accession Q9Y3 M8) STARD13, and the *C. elegans* F25H2.6 (accession CAB02094) putative START protein using the Clustal W algorithm (Thompson *et al.* 1994). Molecular phylogenetic analysis was then performed using the Neighbor Joining method (Saitou & Nei 1987) in MEGA version 3 (Kumar *et al.* 2004). The analysis parameters for the alignment were: pairwise alignment gap opening penalty=10; pairwise alignment gap extension penalty=0.1; multiple alignment gap opening penalty=10; multiple alignment gap extension penalty=0.2; using a Gonnet protein weight matrix with residue-specific and hydrophobic penalties and gap separation distance of 4. Two thousand iterations were used to generate a bootstrap consensus tree, which was rooted with the *C. elegans* F25H2.6 putative START protein.

To determine if amino acid residues and structural motifs important to StAR structure and function are

conserved in the deduced amino acid sequences encoded by the putative *P. motoro* StAR cDNA, this sequence was aligned with StAR proteins from the Japanese eel, rainbow trout, African clawed frog, chicken and human using the Clustal W algorithm (Thompson *et al.* 1994) in the Vector NTI software package (Invitrogen). Computer analysis was also used to predict the secondary structure of the deduced amino acid sequence of *P. motoro* StAR (Kneller *et al.* 1990, McGuffin *et al.* 2000).

Heterologous expression of *P. motoro* StAR

In order to examine the biological activity of the *P. motoro* StAR protein, we cotransfected green monkey kidney (COS-1) cells with *P. motoro* StAR/pCMV-5 and a human cholesterol side chain cleavage/adrenodoxin reductase/adrenodoxin fusion construct (the F2 construct, a generous gift of Dr Walter L Miller, University of California, San Francisco; for details regarding this construct please see Harikrishna *et al.* 1993). Initial studies indicated *P. motoro* StAR mRNA was expressed but poorly translated in mammalian cells. To increase the expression of *P. motoro* StAR in mammalian cells, we used PCR to mutate the wild-type translational start site of the *P. motoro* StAR coding sequence into a completely conserved Kozak's sequence (StAR Kozak S: 5'-GCCA CCATGGTTCCAGCCACTTTTAAGC-3'; Kozak's sequence underlined). In order to facilitate detection of the expressed protein in future studies, an antisense primer (StAR-HSV AS: 5'-TTCCGGGGCGAGTTC TGGCTGGCTGTAAGTGTTC-3') that destroys the wild-type 'stop' signal and introduces the first 24 nucleotides of the sequence encoding the 12 amino acid herpes simplex virus tag (HSV-tag) antigen was used in this initial PCR reaction. This reaction was diluted 1:100 and used as template for a second round of PCR with the StAR Kozak sense primer and an HSV-tag specific AS primer (HSV AS: 5'-AAGCTTCTAGAC TAATCCTCGGGGTCTTCCGGGGCGAG-3') that includes the last 24 nucleotides encoding the HSV antigen (overlapping 12 nucleotides of the StAR-HSV AS primer) followed by a stop signal (TAG) and HindIII and XbaI restriction sites. A high-fidelity thermostable polymerase was used in these reactions (Invitrogen Platinum Taq High Fidelity). PCR products were ligated into pCR II (Invitrogen) and sequenced to confirm sequence fidelity. Confirmed *P. motoro* StAR/pCR II constructs were digested with EcoRI and the liberated StAR insert was gel purified and ligated into EcoRI-digested pCMV5 vector. *E. coli* harboring pCMV5 constructs containing the StAR insert in the proper orientation were identified using a pCMV5-specific sense primer and a *P. motoro* StAR-specific antisense primer. The pCMV/*P. motoro* StAR construct was purified from an overnight culture and used to

transfect COS-1 cells using a commercial transfection reagent (*TransIT-COS*; Mirus Bio Corporation, Madison, WI, USA). COS-1 cells were plated into six-well plates to yield 60–80% confluency the following day, when two wells each were transfected with either empty pCMV5 (0.5 µg), empty pCMV5 (0.25 µg) and F2 (0.25 µg), or pCMV5/*P. motoro* StAR (0.25 µg) and F2 (0.25 µg). All wells also received 0.1 µg CMV/β-galactosidase reporter construct. Cells received fresh media 24 h after transfection, which was subsequently collected 24 h later. Pregnenolone was measured in these samples using a commercial ELISA as per manufacturer's instructions (Diagnostics Biochem, Canada). Following collection of the media, cells were lysed in 250 µl lysis buffer and β-galactosidase activity determined using a commercial kit (Promega) to establish transfection efficiency. Rates of pregnenolone synthesis normalized to β-galactosidase activity were log transformed and analyzed using ANOVA followed by Tukey's post-hoc test. Comparisons generating *P* values less than 0.05 were considered significantly different.

Northern blot analysis

P. motoro interrenal gland total RNA (10 µg) was denatured in 20 mM 3-[*N*-morpholino]propanesulfonic acid, 2 mM sodium acetate and 1 mM EDTA; pH 7.0 (MOPS buffer) with 2.5 M formaldehyde and 50% formamide. RNA was heated at 55 °C for 60 min and chilled in ice water before adding gel-loading buffer (10 × gel loading buffer: 50% glycerol, 10 mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol FF). Standards (5 µg Invitrogen 240–9500 bp RNA ladder) and samples were size fractionated by gel electrophoresis in a 1.5% agarose gel containing 1 × MOPS buffer and 2.2 M formaldehyde for 2.5 h (Sambrook *et al.* 1989). RNA was transferred to a Nytran SuPerCharge membrane using downward capillary action and cross-linked to the membrane by a 6 min exposure to UV light (254 nm). The membrane was prehybridized in North2South (Pierce) hybridization solution for 0.5 h and hybridized for 2 h with horseradish peroxidase-labeled probe generated by excising the full open reading frame of *P. motoro* StAR from the pCMV5 expression construct. The membrane was then washed three times in 2 × standard sodium citrate (0.3 M sodium chloride, 0.03 M sodium citrate; pH 7.0), 0.1% SDS for 5 min, then three times in 2 × standard sodium citrate for 5 min. All hybridizations and washes were conducted at 60 °C in a rotating hybridization oven. The membrane was then immersed in North2 South luminol/enhancer reagent (Pierce) for 5 min at room temperature, wrapped in plastic and exposed to autoradiography film for 1 min.

Expression of StAR in *P. motoro* tissues

RT-PCR was used to determine the tissue distribution of StAR expression in *P. motoro*. Total RNA, isolated from cerebellum, hypothalamus, pituitary, telencephalon, atrium, ventricle, gill, kidney, pancreas, spiral valve, liver, kidney, muscle, interrenal gland, ovary and testis was primed with random hexamers and used as template for RT as described above. These RT reactions were then used in PCR using the same primers used to generate the *P. motoro* StAR/pCMV5 expression construct described above. The thermal parameters for PCRs to determine tissue-specific StAR expression were: 95 °C for 2 min followed by 35 cycles of 30 s at 95 °C, 30 s at 60 °C and 60 s at 72 °C. The quality of each RT reaction was assessed with PCR primers designed to amplify a 1100 bp fragment of *P. motoro* 18S RNA (sense: 5'-GGTACAGTGAAACTGCGAATGG-3'; antisense: 5'-TGGTGCCCTTCCGTCAATTCC-3') using the same thermal parameters. To ensure that the PCR products were not the result of genomic contamination, we performed reverse transcriptase-negative RT-PCR and PCR using *P. motoro* genomic DNA as template.

Results

Isolation of *P. motoro* StAR sequences

The degenerate primers used in initial RT-PCR reactions were designed to amplify a 340 bp fragment of StAR; a product of this size was obtained using *P. hystrix* interrenal gland RNA as template (data not shown). BLAST analysis (<http://www.ncbi.nlm.nih.gov/BLAST/>) of this PCR product found similarity to other StAR sequences. Subsequent RACE reactions using primers designed to this sequence yielded the sequence of most (if not all) of the *P. hystrix* StAR transcript (data not shown, full sequence has been deposited in Genbank; accession no. AY553722). This transcript harbors an open reading frame beginning with a sequence (AGACACAAGATGATTCCA; initiation codon in *italics*) that agrees well with translational start motifs found in less derived vertebrates (Smutzer & Chamberlin 1998). This open reading frame is 852 bp in length and encodes a protein of 284 amino acids that contains a putative START domain, as indicated in BLAST analysis (Marchler-Bauer & Bryant 2004).

RT-PCR and RACE reactions using *P. hystrix* and *P. motoro* StAR primers and *P. motoro* interrenal gland RNA as template yielded an open reading frame of 852 bp that begins with the same translational start sequence found in *P. hystrix* StAR transcripts and encodes a protein that differs from the *P. hystrix* protein only at position 39, which is occupied by a lysine residue in *P. hystrix* and an arginine residue in *P. motoro*.

Computer analysis of the freshwater stingray StAR deduced amino acid sequence

Phylogenetic analysis of the putative freshwater stingray StAR and 32 other START proteins showed seven distinct protein families, STARD4, STARD5, STARD6, STARD10, STARD13, MLN64 and StAR. Freshwater stingray StAR proteins clearly segregate with other StAR proteins (Fig. 1). The putative freshwater stingray StAR proteins are almost identical in size to StAR proteins from other vertebrate classes and amino acid residues important to StAR function and regulation are also highly conserved (Fig. 2). Computer analysis (Kneller *et al.* 1990, McGuffin *et al.* 2000) of the deduced amino acid sequence of *P. motoro* StAR indicates a predicted secondary structure that is very similar to that of human StAR (not shown).

Heterologous expression and activity of freshwater stingray StAR

In addition to structural similarities, *P. motoro* StAR is similar in function to other known StAR proteins (Fig. 3). COS-1 cells transfected with the F2 construct produced 2.92 ± 1.14 ng pregnenolone/h per well (mean of two wells from three independent transfections $\pm$ S.E.M.). Coexpression of F2 with a pCMV5/*P. motoro* StAR construct significantly ($P < 0.01$) increased the synthesis of pregnenolone to 47.36 ± 19.06 ng pregnenolone/h per well. COS-1 cells transfected with empty pCMV5 produced 0.17 ± 0.01 ng pregnenolone/h per well, significantly less than either F2-transfected cells ($P < 0.05$) or F2+StAR-transfected cells ($P < 0.001$).

Characterization of *P. motoro* StAR mRNA expression

Northern blot analysis revealed a single transcript of approximately 4000 bp in *P. motoro* interrenal gland RNA (Fig. 4), slightly larger than the longest *P. hystrix* and *P. motoro* cDNAs isolated by RACE. The mRNA sequences encoding different START proteins within a species are not very similar. For example, when the StAR open reading frame is aligned with the open reading frame of the closely related MLN64, there is only 40% similarity between the human StAR and MLN64 sequences and only 33% similarity between the mouse StAR and MLN64 sequences. Therefore, given the lack of similarity between sequences encoding START proteins within species and the stringent hybridization conditions employed in this study, we are confident that the single band in our Northern blot analysis represents the mRNA encoding *P. motoro* StAR.

StAR expression appears to be more robust in *P. motoro* interrenal gland of both sexes than in other tissues

Figure 1 Molecular phylogeny of START proteins. The deduced amino acid sequences of *P. hystrix* and *P. motoro* StAR proteins were aligned with *Salvelinus fontinalis* (brook trout), *Oncorhynchus mykiss* (rainbow trout), *Anguilla japonica* (Japanese eel), *Danio rerio* (zebrafish), *Gadus morhua* (Atlantic cod), *Xenopus laevis* (African clawed frog), *Gallus gallus* (chicken), *Taeniopygia guttata* (zebrafinch), *Mus musculus* (mouse), *Mesocricetus auratus* (hamster), *Rattus norvegicus* (rat), *Equus caballus* (horse), *Sus scrofa* (pig), *Ovis aries* (sheep), *Bos taurus* (cow) and *Homo sapiens* (human) StAR proteins, *C. elegans*, zebrafish (predicted protein obtained from zebrafish database), *Xenopus tropicalis*, mouse and human MLN64, mouse and human STARD4, mouse and human STARD5, mouse and human STARD6, mouse and human STARD10, and mouse and human STARD13. The tree was rooted using the *C. elegans* F25H2-6 putative START protein. Please refer to Materials and methods for database accession numbers. Molecular phylogenetic analysis was then performed using the Neighbor Joining method (Saitou & Nei 1987) in MEGA version 3 software (Kumar *et al.* 2004) and a bootstrap consensus tree generated. The numbers at branch points represent bootstrapping values obtained with 2000 bootstrap replicates. Branches not supported by bootstrapping values of at least 50% were condensed to produce a multifurcating tree. Freshwater stingray StAR proteins segregate with and assume a position basal to other StAR proteins.

Figure 2 Alignment of the deduced amino acid sequence of freshwater stingray StAR proteins with other StAR proteins. The deduced amino acid sequence of *P. motoro* StAR was aligned to StAR sequences from brook trout, African clawed frog, chicken and human StAR (please refer to Materials and methods for database accession numbers). The deduced amino acid sequence of *P. hystrix* StAR differs from *P. motoro* StAR only at position 39 (lysine in *P. hystrix*; arginine in *P. motoro*). Amino acid residues that are identical in all StAR proteins are white with black background. Dashes indicate spaces introduced to facilitate alignment. Amino acid residues that are known to disrupt STAR function when mutated are indicated by arrows (Stocco 2002), while arrowheads indicate potential sites of serine phosphorylation that may be important to the regulation of STAR activity (Arakane *et al.* 1997). Importantly, computer analysis (Marchler-Bauer & Bryant 2004) identified a putative START domain between residues 72 and 275 of the *P. motoro* StAR protein.

(Fig. 5). In female *P. motoro*, StAR expression was also detected in the atrium, ventricle, gill, muscle and ovary. StAR transcripts can be detected in pituitary and telencephalon samples after 40 PCR cycles (not shown). In male *P. motoro*, StAR mRNA was detected in atrium, ventricle and testis. A weak band appears in the pituitary after 40 PCR cycles (not shown). StAR mRNA was not detected in *P. motoro* cerebellum, hypothalamus (Fig. 5), spiral valve, pancreas, liver or kidney (not shown). Templates used in these reactions were found to be of similar quality as determined by amplification of 18S sequence (Fig. 5). No amplification of StAR occurred in

PCR reactions using either *P. motoro* genomic DNA or reverse transcriptase-negative RT reactions as template (data not shown), which indicates that genomic DNA did not serve as template in StAR RT-PCRs.

Analysis of the UTRs of freshwater stingray StAR transcripts

In *P. hystrix*, the longest 5'-RACE products yielded only 38 bp of 5'-UTR. The *P. hystrix* StAR 3'-UTR is 2472 bp in length (excluding the poly-A tail) and contains a consensus polyadenylation signal (AATAAA)

Figure 3 Heterologous expression of *P. motoro* StAR in COS-1 cells. Green monkey kidney cells (COS-1) transfected with a human side chain cleavage/adrenodoxin reductase/adrenodoxin fusion construct (the F2 construct (Harikrishna *et al.* 1993)) and a *P. motoro* StAR/pCMV-5 expression construct produced pregnenolone at a significantly higher rate (47.36 ± 19.06 ng pregnenolone/h per well) than cells transfected with F2 alone (2.92 ± 1.14 ng pregnenolone/h per well). COS-1 cells transfected with empty pCMV-5 produced significantly less pregnenolone (0.17 ± 0.01 ng pregnenolone/h per well). All cells were transfected with equal amounts of a β -galactosidase expression construct and rates of pregnenolone synthesis were normalized to β -galactosidase activity to control for transfection efficiency. Bars represent the means of six wells from three independent transfections and error bars represent S.E.M. A single asterisk indicates a significant difference ($P < 0.05$) from pCMV5-transfected cells; a double asterisk indicates a significant difference ($P < 0.01$) from pCMV5-transfected cells and a double dagger indicates a significant difference ($P < 0.001$) from pCMV5+F2-transfected cells, as determined by one-way ANOVA followed by Tukey's post-hoc test.

20 bp upstream of a poly-A tail. As in *P. hystrix*, the longest *P. motoro* 5'-RACE products yielded 38 bp of 5'-UTR sequence. RT-PCR and 3'-RACE yielded two overlapping *P. motoro* StAR 3'-UTR sequences (the full sequences have been deposited in Genbank; accession numbers AY553721 and AY553723). The shorter *P. motoro* StAR 3'-UTR is 379 bp in length (excluding the poly-A tail) and includes an atypical polyadenylation signal (CATAAA) 13 bp from the poly-A tail. The longer *P. motoro* StAR 3'-UTR (2472 bp in length excluding the poly-A tail) completely overlaps and extends the shorter transcript. A consensus polyadenylation signal (AATAAA) is found 19 bp from the poly-A tail in this transcript. The 3'-UTR does not harbor additional open reading frames of greater than 60 codons. The 3'-UTR of the *P. motoro* was scanned for possible structural and regulatory motifs using RNA analysis software (<http://wb2x01.biozentrum.uni-wuerzburg.de/>) (Bengert & Dandekar 2003), but no such motifs were detected. However, BLAST analysis of the long 3'-UTR revealed the presence of a sequence very similar to a short interspersed repetitive element (HE1 SINE) found in

Figure 4 Northern blot analysis of StAR expression in freshwater stingray interrenal gland. Northern blot analysis (lane A) of *P. motoro* interrenal gland total RNA indicates a single StAR transcript of approximately 4000 bp. The positions of RNA standards are shown to the left of lane A and a negative image of the pretransfer ethidium bromide-stained interrenal gland RNA is shown in lane B.

elasmobranchs and other vertebrates (Ogiwara *et al.* 1999). Compared with other elasmobranchs, the putative *P. motoro* StAR 3'-UTR SINE sequence is 39% and 48% identical to those of *Mustelus manazo* and *Scyliorhinus torazame* respectively (Fig. 6).

Discussion

Our molecular phylogenetic analysis of 34 START proteins clusters the *P. hystrix* and *P. motoro* proteins with

Figure 5 Expression of StAR mRNA in *P. motoro* tissues. RT-PCR revealed the expression of StAR in multiple tissues from male and female *P. motoro*. In addition to classic steroidogenic tissues such as the interrenal gland and gonadal tissues, StAR mRNA is also detected in the heart of both sexes and in the gill and muscle of female *P. motoro*. Forty PCR cycles were necessary to detect StAR mRNA in the brain. StAR mRNA was not detected in kidney, liver, spiral valve and pancreas of *P. motoro*. Primers designed to amplify approximately 1100 bp of 18S RNA were used in separate RT-PCR reactions to ensure template quality. The position of the 1000 bp DNA standard is indicated by the arrows to the left of each image. Reverse transcriptase-negative reactions and reactions using *P. motoro* genomic DNA as template were used to ensure StAR amplicons were not amplified from contaminating genomic DNA (not shown).

the StAR family. The START protein with the greatest sequence similarity to StAR is MLN64, but MLN64 proteins cluster separately from StAR proteins. The freshwater stingray StAR proteins are also of similar size and show high identity (56–64%) when compared with other StAR proteins. The amino acid residues that are critical to StAR function and regulation (e.g. the serine phosphorylation motif at Ser195) are highly conserved between freshwater stingray StAR proteins and other StAR proteins. In addition, the predicted secondary structures of *P. motoro* and human StAR are similar, with an N-terminal amphipathic helix that may serve as a mitochondrial targeting sequence (Kallen *et al.* 1998). Most of the remaining protein consists of alternating alpha helices and beta strand motifs thought to form the START domain, a hydrophobic tunnel that can bind to a single molecule of cholesterol (Tsujishita & Hurley 2000). Moreover, the *P. motoro* StAR protein can facilitate the synthesis of pregnenolone

by SCC, as indicated by transfection assays in COS-1 cells cotransfected with the F2 human SCC electron transport chain construct. The fusion of a green fluorescent protein (GFP) to the C-terminus of ovine StAR significantly lowers steroidogenic activity (West *et al.* 2001). The addition of a C-terminal HSV-tag might have similar effects on the activity of the *P. motoro* StAR protein. However, the HSV-tag (12 amino acids) is significantly smaller than GFP (238 amino acids); we therefore expect the HSV-tag has minimal effects on StAR activity. In any case, the *P. motoro* StAR/HSV-tag fusion protein has significant steroidogenic activity.

Like StAR, MLN64 can facilitate steroidogenesis; however, MLN64 proteins (about 450 residues) are much larger than StAR proteins (about 280 residues), are not typically targeted to the mitochondria (Moog-Lutz *et al.* 1997, Watari *et al.* 1997), and are less similar to the deduced freshwater stingray proteins (19–57%). These

Figure 6 The long form of the *P. motoro* StAR transcript contains a short interspersed repetitive element. BLAST analysis revealed significant homology between *P. motoro* StAR 3'-UTR sequence 1878–2161 bp and the HE1 family of short interspersed repetitive elements (SINEs) found in the gummy shark (*Mustelus manazo*; Genbank accession no. AB027719), the cloudy cat shark (*Scyliorhinus torazame*; Genbank accession no. AB027721) and other elasmobranch species (Ogiwara *et al.* 1999). The positions of the tRNA-related (dashed line) and central conserved (solid line) regions commonly found in SINEs are indicated above the nucleotide sequence.

findings strongly support the conclusion that we have isolated cDNAs encoding *P. hystrix* and *P. motoro* StAR.

A single, large StAR transcript (approximately 4000 bp) was detected in *P. motoro* interrenal gland, slightly larger than the longest cDNA clone isolated in this species (3365 bp). Repeated attempts to extend the 5'- and 3'-UTRs did not yield additional sequence. While it is possible that we are still lacking untranslated sequence, it is also likely that the difference between the results of northern blot analysis and the length of isolated RACE clones is due to polyadenylation, because poly-A tails can be several hundred nucleotides in length (MacDonald & Redondo 2002). The majority of the *P. motoro* StAR transcript is 3'-UTR sequence, which contains three consensus polyadenylation signals (beginning at 2125, 2130 and 3341 bp). Other elasmobranch steroidogenic enzymes have exceedingly long 3'-UTR sequences with multiple polyadenylation signals. For example, southern stingray (*Dasyatis americana*) SCC is encoded by a large mRNA that includes 3000 bp of 3'-UTR with four consensus polyadenylation signals (Nunez & Trant 1997). Similarly, *D. americana*

3 β -hydroxysteroid dehydrogenase mRNA contains at least 1000 bp of 3'-UTR with two consensus polyadenylation signals (Nunez & Trant 1998).

These differences in 3'-UTR length may have an influence on the function of translated enzymes. For example, *D. sabina* aromatase is encoded by two overlapping transcripts (1700 and 3100 bp) that apparently result from the use of different polyadenylation signals; when these transcripts were expressed in COS cells, the 1700 bp form produced twice the aromatase activity of the 3100 bp form (Ijiri *et al.* 2000). The short *P. motoro* StAR transcript (1324 bp) isolated by 3'-RACE may result from the use of an atypical polyadenylation signal (CATAAA) starting at 1306 bp. StAR transcripts of multiple sizes are common in other vertebrates, including zebrafish (1500, 2500 and 4500 bp), rainbow trout (2300, 4300 and 9900 bp) and humans (1600, 4400 and 7500 bp) (Sugawara *et al.* 1995b, von Hofsten *et al.* 2002, Li *et al.* 2003). The rapid induction of steroidogenesis by peptide hormones and other factors depends on the translation of StAR mRNA, but not on StAR gene transcription, which implies that a significant pool of

StAR mRNA must be maintained in steroidogenic cells (Stocco & Clark 1996). The extensive 3'-UTRs present in StAR and other steroidogenic mRNAs may stabilize these molecules, thus allowing a ready pool of mRNA that can be rapidly translated to augment steroidogenic capacity. The poly-A tail of mRNA and the poly-A binding proteins that bind it are known to protect mRNAs from degradation by exonucleases (for review see Day & Tuite 1998). Lengthy 3'-UTR sequences with multiple polyadenylation signals could act as a buffer against degradation, perhaps further extending the half-life of these mRNA transcripts (Littauer & Soreq 1982, Leff *et al.* 1986).

Analysis of the 3'-UTR of freshwater stingray StAR transcripts indicates the presence of a well-conserved elasmobranch HE1 SINE (Ogiwara *et al.* 1999). To the best of our knowledge, at least one example of an interspersed repetitive element altering the expression of a steroidogenic enzyme has been published; i.e. a retroviral long terminal repeat was discovered in the aromatase promoter of chicken strains that display the henney-feathering trait (Matsumine *et al.* 1991, McPhaul *et al.* 1991). This promoter is apparently responsible for the extragonadal expression of aromatase in these chickens. Although speculative, it is possible that the SINE sequence in freshwater stingray StAR has important physiological consequences. For example, the transcription of some SINE sequences is induced by cellular stress and high levels of SINE RNA molecules have been shown to inhibit the ability of the double-stranded RNA-dependent protein kinase (PKR) to inhibit translation (Liu *et al.* 1995, Schmid 1998). Other studies have revealed that SINE sequences can activate a PKR-independent pathway that stimulates the translation of newly transcribed mRNA (Rubin *et al.* 2002). StAR SINE sequences could augment translation through induction in response to stress, inhibition of PKR and induction of PKR-independent pathways. The presence of a SINE-like sequence in the freshwater stingray StAR 3'-UTR is of interest because the adrenocortical response to ACTH depends on a rapid increase in StAR translation, and sustained steroidogenesis depends on efficient translation of newly transcribed StAR mRNA (Stocco 2001).

In freshwater stingrays, StAR is expressed in several tissues, including those of the nervous, cardiovascular and reproductive systems. These data are consistent with studies in other vertebrates that demonstrate StAR expression is not restricted to adrenocortical and gonadal tissue. For example, in mammals, StAR mRNA has been detected in human fetal and adult kidney (Sugawara *et al.* 1995a), and in glial and neuronal cells of the cerebellum, hypothalamus and other regions of the human brain (King *et al.* 2002). Using northern blot analysis, Bauer *et al.* (2000) detected StAR mRNA in the head of a teleost (zebrafish), but not in the brain of

the chicken; StAR transcripts were also detected in the zebrafish kidney, although this could be due to the inclusion of adrenocortical cells of the head kidney (the interrenal gland of teleosts). StAR expression was detected by northern blot and RT-PCR analysis in rainbow trout pyloric ceca, intestine and spleen in addition to head kidney and gonadal tissue, but only in traditional steroidogenic tissues (head kidney and gonads) in brook trout (Kusakabe *et al.* 2002a).

In freshwater stingrays, the detection of StAR expression in extragonadal and extraadrenocortical tissues, such as the heart, gill and muscle, is significant because these tissues do not express SCC mRNA (B S Nunez and S L Applebaum, unpublished observations) and therefore cannot convert cholesterol to pregnenolone. In contrast, most extragonadal and extraadrenocortical tissues in mammals that express StAR (e.g. brain, heart) also express SCC and are therefore able to produce steroids (King *et al.* 2002, White 2003). StAR activity is not specific to steroidogenesis, but can increase the activity of other cholesterol-metabolizing enzymes such as cholesterol 27-hydroxylase (Sugawara *et al.* 1995a). StAR is expressed in the human kidney, a tissue that does not express SCC, but does produce steroid-like molecules (e.g. vitamin D hormones). The fact that StAR is expressed in tissues such as *P. motoro* heart, muscle and gill, rainbow trout pyloric ceca, intestine and spleen and human (and potentially zebrafish) kidney is an indication that StAR may be important to other biosynthetic pathways that utilize cholesterol. Of particular interest is the synthesis of endogenous cardiac glycosides (e.g. ouabain, digoxin, bufadienolides), cholesterol derivatives which can have both rapid (through interaction with the sodium/potassium-ATPase) and long-term (through the induction of gene expression and cell proliferation) effects on ionoregulation and cardiovascular function (Schoner 2002). Although their biosynthetic pathways have not been fully elucidated (Hamlyn 2004), there is evidence that several enzymes (including SCC) may be common to the synthesis of some cardiac glycosides and corticosteroids (Hamlyn *et al.* 2003). However, the synthesis of bufadienolide in mammals appears to be independent of both StAR and SCC, as indicated by studies using mouse Y-1 adrenocortical cells transfected with genes that inhibit the expression of StAR and SCC (Dmitrieva *et al.* 2000). Recent work suggests that endogenous cardiac glycosides may have an important role in the regulation of ionoregulation in fishes. A significant increase in immunoreactive plasma ouabain was detected in tilapia at 4 and 24 h following transfer from freshwater to seawater and 4 h after transfer from seawater to freshwater (Kajimura *et al.* 2004). This study found high concentrations of ouabain in extracts of several tissues, with the highest concentrations found in the interrenal gland (unfortunately the heart was not

examined). The effects of ouabain in tilapia are dose dependent, as concentrations which do not inhibit sodium/potassium-ATPase (10–100 pM) significantly inhibit the release of prolactin from the tilapia pituitary, while concentrations that inhibit sodium/potassium-ATPase (100–1000 nM) stimulate prolactin release (Kajimura *et al.* 2005). Therefore, while StAR may not have a role in bufadienolide synthesis, there is a good possibility that StAR (or another START protein) is involved in the regulated synthesis of cardiac glycosides.

Acknowledgements

We would like to thank Dr Walter L Miller of The University of California, San Francisco, for the generous gift of the F2 construct and Dr David Foltz of Louisiana State University for assistance with molecular phylogenetic analysis. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 14 September 2005

Accepted 23 September 2005