Seladin-1 expression in rat adrenal gland: effect of adrenocorticotropic hormone treatment

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Abstract

Seladin-1 (KIAA0018) gene is the seventh most highly expressed gene in the adult adrenal gland, along with genes coding for steroidogenic enzymes. The aim of the present study was to investigate the localization of the Seladin-1 protein in control and ACTH-treated rat adrenal glands and to verify whether Seladin-1 is involved in secretion. Immunofluorescence studies revealed that Seladin-1 was localized principally in the zona fasciculata, cytoplasm, and nucleus. Expression of Seladin-1 was increased by ACTH treatment, in vivo and in culture conditions. Subcellular fractionation of fasciculata cells showed that Seladin-1 was mainly present in the nucleus, membrane, and cytoskeleton fractions and, to a lesser extent, in the cytosol. ACTH treatment decreased Seladin-1 expression in the cytosol, with a concomitant increase in the nuclear fraction. In the glomerulosa and fasciculata cells in culture, ACTH induced a relocation of Seladin-1 into specific nuclear regions. This ACTH-induced relocation was abrogated by the pretreatment of cells with 75 nM U18666A (an inhibitor of Seladin-1). In addition, fasciculata cells exhibited an increase in the basal level of steroid secretion when cultured in the presence of U18666A (25 and 75 nM), although ACTH-induced secretion was decreased. In summary, the present study demonstrates that the protein expression of Seladin-1 is more abundant in fasciculata cells than in glomerulosa cells and that the ACTH treatment increases both expression and nuclear localization of the protein. Results also suggest that depending on its cellular localization, the Δ24-reductase activity of Seladin-1 may play a major role in steroid secretion in the adrenal gland.


Introduction

The adult adrenal cortex is composed of three concentric layers: the zona glomerulosa, the zona fasciculata, and the zona reticularis, all of which present different morphological and functional properties. Zona glomerulosa is specialized in the production of aldosterone while zona fasciculata/reticularis synthesize cortisol in humans and bovine, and corticosterone in rodents (for review, see Rainey 1999). The overall production of aldosterone, however, is in the order of picomolar, compared with the micromolar range for cortisol/corticosterone (Gallo-Payet & Payet 1989, Rainey 1999, Sewer & Waterman 2003). The adrenal gland undergoes constant dynamic structural changes and is generally well acknowledged that cellular proliferation is preferentially observed at the periphery of the gland (zona glomerulosa) while cell death is increased in zona reticularis. The regulated balance between proliferation and apoptosis is a prerequisite for the integrative functionality of the gland (for review, see Wolkersdorfer & Bornstein 1998, Vinson 2003, 2004).

Aside from angiotensin II (Ang II), adrenocorticotropic hormone (ACTH) is the most potent stimulus of aldosterone secretion by glomerulosa cells and of corticosterone by fasciculata cells (Gallo-Payet & Payet 2003). ACTH acts not only on the immediate, transcription-independent stimulation of adrenal steroid synthesis and release, but also increases the expression of a number of genes including those involved in steroidogenesis (Sewer & Waterman 2003). Despite several studies using both animal and human glomerulosa and fasciculata cells, the precise molecular mechanisms by which ACTH stimulates growth and secretory activities are complex and poorly understood. Over the past few years, studies on gene profiling and regulation have provided key elements in our comprehension as to how cell functions are regulated at the molecular level. For instance, Seladin-1 (KIAA0018) gene is the seventh most highly expressed known gene in the human adult adrenal gland, along with genes coding for steroidogenic enzymes (Hu et al. 2000). This gene is also expressed in the human fetal adrenal gland with a fourfold increase in the expression when compared with the adult (Rainey et al. 2001), thus being the highest expressed known gene in human fetal adrenal gland (Rainey et al. 2002). This protein has also been identified in the brain and termed Seladin-1, for SELective Alzheimer Disease INdicator 1, due to its propensity to protect neurons from β-amyloid peptide-induced toxicity, thus promoting cell survival.
Seladin-1 is a human homolog of the Diminuto/Dwarf1 gene described in plants where it is involved in growth and steroid synthesis (Takahashi et al. 1995, Klahre et al. 1998). In normal human adrenal cortex, Seladin-1 mRNA expression is present throughout the gland although more intense in the zona fasciculata (Sarkar et al. 2001). In adrenal glands from dexamethasone-treated rats, Seladin-1 mRNA expression is present in a reduced form of ACTH in adrenal carcinomas (Sarkar et al. 2001, Luciani et al. 2004). The human adrenocortical cancer cell line H295R as well as in the cultured human primary adrenocortical cells, Seladin-1 mRNA is regulated by the ACTH/cAMP pathway (Sarkar et al. 2001, Luciani et al. 2004).

Seladin-1, a member of the flavin adenine dinucleotide-dependent oxidoreductases family, is also named 3-β-hydroxysterol Δ-24-reductase (DHCR24; Greeve et al. 2000). This enzyme is known to catalyze the conversion of desmosterol to cholesterol (Waterham et al. 2001), the preferential pathway of cholesterol synthesis in the human and the rat fetal brain (Fumagalli & Paoletti 1963). In humans, mutations of the DHCR24 gene result in a rare and severe recessive autosomal disorder called desmosterolosis. This pathology is characterized by desmosterol accumulation in plasma and tissues, by multiple congenital anomalies, and by severe mental retardation (Waterham et al. 2001). Recently, Seladin-1 was also described as a key regulator of Ras-induced senescence and responses to oncogenic and oxidative stress (Wu et al. 2004).

There is limited data available relative to Seladin-1 at the protein level in the adrenal gland, the tissue with the highest transcription level of Seladin-1 (Greeve et al. 2000). Therefore, the first aim of the present study was to generate a specific antibody to investigate the expression and cellular/intracellular distribution of Seladin-1 protein in the adrenal gland of both control and ACTH-treated rats. The second aim was to confirm this distribution in primary cultures of adult rat glomerulosa and fasciculata cells, before and after ACTH treatment. Using this model, we thereby explored whether Seladin-1 is possibly involved in selected adrenocortical cell functions.

Materials and Methods

Chemicals

The chemicals used in the present study were obtained from the following sources: Tissue-Tek from Miles (Elkhart, IN, USA); Minimum Essential Medium (MEM Eagle’s medium) and OPTI-MEM® from Life Technologies; collagenase from New England Nuclear (Boston, MA, USA); DNase from Sigma Chemical Co.; ACTH 1–24 peptide (Cortrosyn) from Organon (Toronto, Ont., Canada); ACTH 1–39 peptide (Synacthen Depot 100 IU/ml) from Ciba Pharmaceuticals (Caldwell, NJ, USA); angiotensin II from Bachem (Marina Delphen, CA, USA). BrdU (5-bromo-2-deoxyuridine), anti-BrdU, the secondary antibodies (mouse and rabbits) coupled to Alexa-Fluor-488, Alexa-Fluor-594, the lectin wheat germ agglutinin (WGA), and 4',6'-diamino-2-phenylindole (DAPI) were from Molecular Probes (Eugene, OR, USA). The anti-actin was from Chemicon International, Inc. (Temecula, CA, USA); Vectamount from Vector Laboratories (Burlingame, CA, USA); horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies and enhanced chemiluminescence (ECL) system from Amersham. The ProteoExtract Subcellular Proteome Extraction Kit and the Seladin-1 inhibitor, U18666A (3β-(2-diethylaminoethoxy)androst-5-en-17-one), were purchased from Calbiochem (San Diego, CA, USA) and the RNAaqueous-4PCR Kit from Ambion (Austin, TX, USA). [3H]aldosterone (76 Ci/mmol) and [3H]corticosterone (70 Ci/mmol) were obtained from New England Nuclear, Dupont (Boston, MA, USA) and the anticorticosterone from Medicorp (Montreal, Quebec, Canada). Aldosterone antiserum was purchased from ICN Biochemicals (Costa Mesa, CA, USA). All the other chemicals were of A-grade purity.

Animals

Long–Evans female rats weighing 200–250 g were purchased from Charles River, St Constant, Quebec, Canada. Upon arrival, the rats were kept under controlled light and temperature in our institution’s animal care facilities for 2 weeks prior to the start of the experimental procedure to allow the animals to adapt to their environment, thereby reducing stress. Rats were then randomly distributed in control or in ACTH-treated groups. Animals were treated once a day (1500 h) for 7 days either with saline (control) or with Synacthen Depot subcutaneously (8 IU/250 g body weight per day) and killed by decapitation on the morning of the eighth day. All the protocols were approved by the Animal Care and Ethics Committee of our faculty. A number of three to five animals were used for each experiment and three different experiments were performed.

Generation and characterization of Seladin-1 antiserum

The Seladin-1 antiserum was produced by Affinity Bioreagents (Golden CO, USA): rabbits were immunized with the same peptide as the one used by Greeve et al. (2000), consisting of amino acid residues 203–218 of Seladin-1 H3N-TPSENSDLFYAVPWSC-COOH, maleimide-conjugated to keyhole-limpet hemocyanin. This sequence is identical in human, rat, and mouse. Specificity of the antibody was confirmed by western blotting. Briefly, following electrophoresis and transfer, polyvinylidene difluoride membranes were incubated as described below with Seladin-1 antiserum (dilution 1:1000), with the pre-immune serum (dilution 1:1000) or with the Seladin-1 antiserum neutralized by the peptide raised against at 175-fold molar excess. The
expression of Seladin-1 was compared with total rat adrenal gland, brain, and cerebellum for equal protein loading (20 μg). Seladin-1 antiserum detected two bands (77 and 47 kDa) in the adrenal gland (lane 2), and three bands (77, 60, and 47 kDa) in the brain (lane 3) and cerebellum (lane 4; Fig. 1A). Lane 5 shows a negative control (Laemmli buffer). Since the pre-immune serum (Fig. 1B) detected one band in the adrenal gland (47 kDa) and two bands in the brain and cerebellum (60 and 47 kDa), these were deemed non-specific bands. Therefore, the 77 kDa band observed in Fig. 1A appeared as specific for Seladin-1. With the peptide neutralized-Seladin-1 antiserum, the 77 kDa band was no longer detected in the three tissues (Fig. 1C), indicating specificity of this band. Similar observation was also made in the human fetal adrenal gland (data not shown).

Preparation of glomerulosa and fasciculata cell cultures

Glomerulosa and fasciculata cells were obtained from adrenal glands of female Long–Evans rats and isolated according to the method previously described in detail (Gallo-Payet & Payet 1989). Isolation and cell dissociation of glomerulosa and fasciculata zones were performed in MEM (supplemented with 2% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin). After a 20-min incubation at 37 °C with collagenase (2 mg/ml) and DNase (25 g/ml), cells were disrupted by gentle aspiration with a sterile 10 ml pipette, filtered, and centrifuged for 10 min at 100 g. For western blot analyses (Fig. 6) from freshly isolated cells, 1 x 10⁶ glomerulosa and fasciculata cells were immediately processed for subcellular protein extraction, and frozen until western blotting. For cell cultures, cell pellets were resuspended in OPTI-MEM® supplemented with 2% fetal bovine serum, 100 U/ml penicillin, and 100 g/ml streptomycin. The glomerulosa and fasciculata cells were plated in triplicate for immunofluorescence experiments (5 x 10⁴ cells per dish). The cells were left to adhere to the Petri dishes for 45 min before adding the culture medium. The cells were cultured at 37 °C in a humidified atmosphere composed of 95% air–5% CO₂. The culture medium was changed 6 h after initial addition of the medium, and the cells were used after 3 days of culture. The glomerulosa cells were stimulated with either Ang II (100 nM) or ACTH (10 nM) for 3-day treatments, twice a day and fasciculata cells were stimulated with ACTH (10 nM) for 3-day treatments, also twice a day.

Fasciculata cells (5 x 10⁴ cells) were also treated for 3 days with or without ACTH (10 nM, twice a day) alone or in the presence of the Seladin-1 inhibitor, U18666A (25–75 nM, once a day). U18666A, a cell-permeable, amphiphilic amino-steroid, is a high-affinity binding inhibitor of the Δ24-reductase activity of Seladin-1 (IC₅₀: 0.15 μM), resulting in desmosterol accumulation. At low concentrations (nanomolar range), U18666A inhibits the cholesterol metabolism activity of Seladin-1. The cells were examined daily, and phase-contrast images were taken using a microscope (Leica Corp., Deerfield, IL, USA) equipped with a ×32 objective.

Immunofluorescence studies in whole glands and cultured cells

Control and 7-day ACTH-treated female Long–Evans rats were killed and adrenal glands dissected and retrieved. Glands were either embedded in paraffin for hematoxylin–eosin

Figure 1 Representative western blot analysis of Seladin-1 expression. (A) Seladin-1 antiserum detection in rat adrenal gland (lane 2), rat brain (lane 3), and rat cerebellum (lane 4) for equal protein loading (20 μg). Lane 1 shows kaleidoscope markers and lane 5 serves as a negative control (Laemmli Buffer). In the adrenal gland, both the 77 and the 47 kDa bands are present while in the brain and cerebellum; there is an additional band at 60 kDa. (B) Immunoblot in the presence of pre-immune serum. In the adrenal gland, only the 77 kDa band is specific since the 60 and 47 kDa bands are also detected with the pre-immune serum. (C) Peptide neutralization assay confirming the specificity of the 77 kDa band, since the latter disappears in all three tissues. All the assays were performed at identical dilutions and exposure times.
counterstaining in order to identify morphological structures or frozen and embedded in a cryoprotectant optimal cutting temperature (OCT Tissue–Tek) for immunofluorescence studies. Tissue sections (5 μm) or glomerulosa and fasciculata cells grown in culture for immunofluorescence were fixed in 3-7% formaldehyde, permeabilized in PBS–0-2% Triton X-100, and blocked in PBS–0-5% BSA, 0-2% Triton. Tissue sections and cells were then incubated with the primary anti-Seladin-1 antiserum or with the pre-immune serum (both 1:1000) followed by incubation with a secondary conjugated anti-rabbit antibody coupled with Alexa-Fluor-594 nm (1:500; red, frozen tissue) or Alexa-Fluor-488 nm (1:500; green, cultured cells) for 1 h at room temperature. The cells were also incubated with the lectin WGA, known as a Golgi apparatus marker (5 μg/ml; red) coupled to Alexa-594 (1:500, red; Kovacs et al. 2004). Tissues and cells were also stained with DAPI (1:1000; blue) for visualization of nuclei. Slides were mounted with Vectashield mounting medium and images were acquired with a Hamamatsu, ORCA-ER digital camera and examined under a fluorescence Nikon Eclipse 2000 inverted microscope (Nikon, Mississauga, ON, Canada) equipped for epi-illumination. Tissue images were acquired using a ×10 or ×20 objectives, whereas cell images were acquired using a ×100 objective. Images were processed with Metamorph (version 4.6r10) software (Universal Imaging Corporation, Downingtown, PA, USA). In all the cases, no specific staining was observed when primary antiserum was replaced by pre-immune serum at the same dilution and exposure time. Images were acquired using identical camera settings for contrast and brightness. To better evaluate Seladin-1 nuclear expression within the zona glomerulosa (under the capsule, four to five cell layers), zona fasciculata, and zona reticularis, results were expressed as the percentage of Seladin-1-stained nuclei found within each cortical zone when compared with the total number of nuclei present in that zone. Seladin-1 staining within the nuclei was defined as the resulting pink color obtained by the superposition of DAPI and anti-Seladin-1, while total number included blue-colored nuclei.

**Tissue protein extraction and subcellular fractionation**

The glomerulosa and fasciculata zones were isolated from the adrenal gland and separately snap-frozen in liquid nitrogen and stored at −80 °C for tissue protein extraction, or cells were immediately processed for subcellular protein fractionation, as described below. The zona fasciculata fraction also contains the zona reticularis, which is difficult to separate from the medulla which we attempted to discard with two small scalpel cuts, but remnants are always possible. Since this fraction contains predominantly fasciculata, it is referred to as zona fasciculata throughout the text. Total tissue protein extraction was performed after addition of boiling 2% SDS/1% Triton/PBS solution to the zona glomerulosa and zona fasciculata tissues (0·2 g tissue/ml). Tissues were homogenized using a Teflon potter and heated at 100 °C for 5 min. This procedure was repeated thrice. Subcellular protein fractionation (cytosol, membranes, nucleus, and cytoskeleton fractions) was done using the ProteoExtract Subcellular Proteome Extraction Kit according to the manufacturer’s instructions (Calbiochem). This kit contains four extraction buffers prepared with ultra-pure chemicals to ensure high reproducibility, protease inhibitor cocktail to prevent protein degradation and benzonase nuclease to remove contaminating nucleic acids. ProteoExtract Subcellular Proteome Extraction Kit yields the total proteome fractionated into four subproteomes of decreased complexity. This kit has a high efficiency for subcellular fractionation (Abdolzade-Bavil et al. 2004) and is widely used (Singh et al. 2004, Zhang & Insel 2004). All samples were assayed for protein content before western blotting. Equivalent amounts of proteins (15–30 μg for each fraction of subcellular fractionation of isolated cells and 15 μg for total tissue protein extraction) were compared in each experiment.

**Western blotting**

All the samples were separated on 12% SDS-polyacrylamide gels and proteins transferred electrophoretically onto poly-vinylidene difluoride membranes. After two washes with Tris-buffered saline (pH 7·5)—Tween 20 (0·05%; TBS-T), membranes were blocked with 5% milk/TBS-T. Membranes were incubated with Seladin-1 antiserum (dilution 1:1000). Detection was performed by reaction with horseradish peroxidase-conjugated anti-rabbit secondary antibody and visualized by enhanced chemiluminescence (ECL system) according to the manufacturer’s instructions. Immuno reactive bands were scanned by laser densitometry and expressed in arbitrary units.

For whole zona glomerulosa and zona fasciculata protein extracts, since the cell volume of glomerulosa cells is lower than that of fasciculata cells, quantification of blots was normalized to actin. For whole gland lysate immunoblotting, after Seladin-1 incubation, membranes were stripped for 2 h with glycine 0·2 M (pH 2·5) at 70 °C, then washed, blocked in 1% gelatin, and further incubated with the anti-actin antibody for 16 h (dilution 1:1000). In the experiments involving subcellular fractionation, the results are expressed as the percentage of Seladin-1 protein within each subcellular fraction relative to total Seladin-1 content within the cell.

**RNA extraction and quantitative real-time PCR**

Total RNA from glomerulosa and fasciculata zones, obtained from control and ACTH-treated rats, was extracted and treated with DNase I (to digest contaminating genomic DNA) using RNAqueous-4PCR Kit according to the manufacturer’s recommendations. RNA content was measured spectrophotometrically while RNA quality was assessed by electrophoresis on denaturing 1% agarose gel. Total RNA (0·5 μg) was denatured (65 °C, 5 min) in the presence of 0·5 μg oligo(deoxythymidin)12–18 (Invitrogen)
and 0.5 mM dNTP (Amersham Pharmacia Biotech) and reverse transcribed at 42 °C for 60 min in 20 μl of 1× first strand buffer (250 mM Tris–HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂) containing 10 mM dithiothreitol, 40 U RNase OUT inhibitor (Invitrogen), and 200 U SuperScript II Reverse Transcriptase (Invitrogen). Inactivation of the enzyme (70 °C, 15 min) was followed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR to assess the quality of the template cDNA. Real-time PCR primers for rat Seladin-1 and GAPDH (Invitrogen) were designed with Beacon Designer 2.0 software (PREMIER, Biosoft International, Palo Alto, CA, USA). Primer pairs are located on both sides of an intron or on an exon–exon junction. GAPDH: sense, TGTTGCCCCAAGGCTCATC; antisense, CTTCCACGATGCCAAAGTTG; and Seladin-1: sense, GGGTGTGTGTGGCTCTCTCC; antisense, GCTCCTTCCACCTCCGGTACC. Real-time PCR was performed with an iCycler iQ Detection System using iQ SYBR Green Supermix (Bio-Rad Laboratories) according to the manufacturer’s instructions and as described previously (Salzmann et al. 2004). Annealing temperature was 60-4 °C. Real-time PCR, products were analyzed on 1× Tris/acetate/EDTA-buffered 2.5% agarose gel and visualized by ethidium bromide staining. To ensure the specificity of each real-time PCR, prior melting curve analyses were performed for all detected products with only a single peak recorded corresponding to a unique melting temperature specific to each product. Sizes of the amplicons obtained were 176 pb for GAPDH and 156 pb for Seladin-1.

Incubation and measurement of aldosterone and corticosterone secretion

Fasciculata and glomerulosa cells were plated in 24-well plates (50×10³ cells/well) and were treated daily for 3 days with or without ACTH (10 nM, twice a day) alone or in the presence of the Seladin-1 inhibitor, U18666A (25–75 nM, once a day). After 3 days, the incubation medium was removed by aspiration and stored at −20 °C for RIAs. For acute stimulations, on the third day of cell culture, cells were stimulated with or without ACTH (10 nM) alone or in the presence of U18666A (25–75 nM), during a 2-h period. Prior to each experiment, the medium from cultured cells was aspirated, and the cells washed twice with cold Hanks’ buffered saline (HBS; 130 mM NaCl, 3.5 mM KCl, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 2.5 mM NaHCO₃, and 5 mM HEPES) supplemented with 1 g/l glucose and 0.5% BSA. The cells were incubated in a 1 ml solution consisting of 0.9 ml HBS-glucose supplemented with 0.5% BSA–0.1 mg/ml bacitracin and 0.1 ml stimuli. After a 2-h incubation at 37 °C in a 95% air–5% CO₂ environment, the incubation medium was removed by aspiration and stored at −20 °C until assayed for steroid content determined by RIA, using specific antiserum and tritiated steroid as tracer.

Proliferation assays

The cell proliferation was measured using fluorescence BrdU incorporation as previously described (Otis et al. 2005). The glomerulosa and fasciculata cells were plated in 24-well plates (30×10³ cells/well). The cells were treated daily for 3 days with or without ACTH (10 nM twice a day) alone or in the presence of the inhibitor, U18666A (25–75 nM, once a day). On the third day, cells were fixed with 3.7% formaldehyde in HBS for 10 min at room temperature and permeabilized for 10 min with 0.2% Triton X-100 in HBS. The cells were then incubated with anti-BrdU Alexa-Fluor-594 (1:500). Fluorescence intensity was determined using a Microplate Fluorescence Reader FL600 (Bio-Tek; excitation: 560/40 nm; emission: 645/40 nm).

Data analysis

The data are presented as means ± S.E.M. of the number of experiments indicated in parentheses. Statistical analyses of the data were performed using one-way ANOVA, followed by a test of simple effects when appropriate. Homogeneity of variance was assessed by Bartlett’s test and P values were obtained by Tukey honestly significant differences. For simple comparisons between two groups, Student’s t-test was performed.

Results

Localization and protein expression of Seladin-1 in adrenal tissue and cells from control and ACTH-treated rats

Using the Seladin-1 antibody characterized in Fig. 1, the distribution and cellular localization of Seladin-1 were first studied by immunofluorescence on frozen adult rat adrenal glands. As shown in Fig. 2A, the highest expression of Seladin-1 was found at the periphery of the adrenal gland, in the zona glomerulosa and zona fasciculata, while the lowest expression was found in the zona reticularis and medulla. To better differentiate intracellular expression, colocalization with nuclear labeling (Fig. 2B) highlighted that, in zona glomerulosa (Fig. 2B, inset a) and zona fasciculata (Fig. 2B, inset b), labeling was observed in both the nucleus (arrow) and the cytoplasm (arrowhead). However, labeling in the nucleus was greater and stronger in zona fasciculata than in zona glomerulosa. In zona reticularis (Fig. 2B, inset c), nuclear labeling was present but less in the other zones while cytoplasmic labeling was very weak. In the medulla, Seladin-1 labeling was practically absent. A 7-day treatment with ACTH increased overall expression of Seladin-1 throughout the gland (Fig. 2C and D). As shown in corresponding insets (Fig. 2D, insets a, b and c), labeling was increased in the nuclei in the three zones. Labeling was completely abrogated when Seladin-1
Figure 2. Immunofluorescence detection of Seladin-1 in rat adrenal gland. Frozen sections were processed as described in Materials and Methods. Seladin-1 labeling was detected using anti-Seladin-1 (1:1000) antibody coupled to Alexa-Fluor-594 nm (A, C, red) or combined with visualization of nuclei with DAPI (blue) (B, D, merged images) in zona glomerulosa (ZG), zona fasciculata (ZF), zona reticularis (ZR), and medulla (M) of control (A and B) and ACTH-treated rats (8 IU/250 g body weight/day per 7 days) (C and D). The resulting pink color in merged images indicates colocalization of Seladin-1 within the nucleus. Non-specific labeling of Seladin-1 pre-immune serum (E). Insets a, b, and c are magnifications of selected areas of zona glomerulosa (ZG), zona fasciculata (ZF), and zona reticularis (ZR) of B and D. Arrows identify labeling in the nucleus, while arrowheads indicate labeling in the cytoplasm. V, central vein in the medulla. Images were acquired with a ×10 objective. Scale bars, 50 μm for A and B, 150 μm for C and D, 25 μm for E, and 10 μm for insets a, b, and c. *Saturated signal.
Figure 3 Hematoxylin–eosin staining of control (A) and ACTH-treated (8 IU/250 g body weight/day per 7 days) (B) rat adrenal glands from the capsule to the mid-fasciculata zone. Lines delineate zona glomerulosa (ZG) from zona fasciculata (ZF).

Figure 4 Quantification of Seladin-1 immunofluorescence labeling in whole frozen adrenal gland sections. Results represent the percentage of nuclei labeled with Seladin-1 protein relative to the total number of nuclei within each adrenocortical section of zona glomerulosa (ZG), zona fasciculata (ZF), and zona reticularis (ZR) from control (n=3) and ACTH-treated rats (8 IU/250 g body weight/day per 7 days; n=3). Results are expressed as means ± S.E.M. Statistical significance when compared with respective control conditions: *P<0.05.

Seladin-1 localization in rat adrenal glands

M-C Battista and others


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Localization of Seladin-1 in primary cultures of glomerulosa and fasciculata cells

Since Seladin-1 has been described for its involvement in senescence (Wu et al. 2004), it appeared important to use primary cell cultures rather than cell lines or transfected cell lines as a more appropriate tool to better understand its role in cellular functions. Figure 7 illustrates the results from fasciculata cells, although glomerulosa cells also exhibited similar labeling (data not shown). Control cells exhibited both cytoplasmic and nuclear labeling (Fig. 7A), and all the cells responded to ACTH treatment with a Seladin-1 relocalization in specific nuclear regions (Fig. 7B). To better delineate intracellular Seladin-1 localization, studies were focused on single cells. In control conditions, Seladin-1 labeling was localized within the cytoplasm and the nucleus (Fig. 7C). In the cytoplasm, labeling was associated with specific cellular structures. Double immunofluorescence studies with the WGA lectin revealed a colocalization of Seladin-1 with the Golgi apparatus as depicted by the overlapping yellow color in the merged images. In the nucleus, labeling was mostly diffused, except in certain particular foci (Fig. 7C). In ACTH-treated cells, Seladin-1 remained localized to the Golgi, which was more condensed around the nuclei, as well as in the nucleus, concentrated into specific nuclear regions (Fig. 7D).

Quantitative real-time PCR performed on in vivo rat adrenal gland zona glomerulosa (ZG) and zona fasciculata (ZF) from control and ACTH-treated rats (8 IU/250 g body weight/day per 7 days). Glands were processed as described in Materials and Methods. The results indicate the fold difference in Seladin-1 mRNA expression between zona fasciculata versus zona glomerulosa (ZF/ZG ratio) in control (n=3) and ACTH-treated rats in zona glomerulosa and zona fasciculata and the fold increase of Seladin-1 mRNA expression in ACTH-treated versus control rats in zona glomerulosa and zona fasciculata.

Table 1 Expression of Seladin-1 mRNA in zona glomerulosa and zona fasciculata in adrenal glands from control and ACTH-treated rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ZF/ZG ratio</th>
<th>ZG (fold increase)</th>
<th>ZF (fold increase)</th>
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<tbody>
<tr>
<td>Control</td>
<td>2·00</td>
<td>–</td>
<td>–</td>
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<tr>
<td>ACTH</td>
<td>1·91</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ACTH/control rats</td>
<td>–</td>
<td>2·13</td>
<td>2·03</td>
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Statistical significance when compared with respective control conditions: *P<0·05.
However, in ACTH-stimulated cells, Seladin-1 was retained in the Golgi apparatus (Fig. 7F), indicating that pre-incubation with U18666A abrogated the ACTH-induced relocalization of Seladin-1 into specific nuclear regions.

**Effect of Seladin-1 inhibitor (U18666A) on steroid secretion and proliferation**

In order to investigate the possible involvement of Seladin-1 in steroid secretion, glomerulosa and fasciculata cells were treated with or without ACTH alone or with U18666A (25 and 75 nM), either for 2 h or 3 days. Results from Fig. 8 indicate that both 2-h and 3-day treatments with U18666A increased basal steroid output in glomerulosa and fasciculata cells, but decreased ACTH-induced aldosterone and corticosterone secretion. The inhibitory action of U18666A on ACTH-induced steroid secretion was more pronounced however after 3 days than after acute 2-h treatment, and was more pronounced for aldosterone secretion in glomerulosa cells than for corticosterone secretion in fasciculata cells (63 versus 43% reduction respectively). These results suggest that Seladin-1 is differentially implicated in basal and in ACTH-induced steroid secretion. On the other hand, 3-day treatment with U18666A did not modify active cell proliferation observed in cultured cells (data not shown).

**Discussion**

This study characterizes for the first time the expression, distribution, intracellular localization, and dynamics of Seladin-1 protein in adrenal glands and in primary cultures of adrenocortical cells, in control and in ACTH-treated rats. The most notable findings of this study are that Seladin-1 (1) is localized both in the cytoplasm and in the nucleus; (2) subcellular localization is regulated by ACTH; and (3) is differentially involved in basal and in ACTH-stimulated steroid secretion.

In virtually all studies, Seladin-1 has been studied at the mRNA level in transfected or retroviral-transduced cells, under non-physiological stimulations (Greeve et al. 2000, Wu et al. 2004) or *in vivo* in normal and pathological conditions in human (Sarkar et al. 2001, Fuller et al. 2005, Luciani et al. 2005). Herein, the antibody generated to conduct the present investigations revealed a single band with a molecular weight (MW) of 77 kDa band for Seladin-1 in the rat adrenal gland, brain, and cerebellum. This band was not detected when Seladin-1 antiserum was replaced by the pre-immune serum at same dilution and exposure time or when the Seladin-1 antiserum was neutralized by the peptide raised against Seladin-1, indicating specificity of the antibody. A previous study had reported a Seladin-1 MW of 60 kDa in normal and Alzheimer’s disease brain tissues from 62- to 92-year-old patients (Greeve et al. 2000). The differences between these values may be related to differences in species (human versus rat) or age. However, we cannot rule out the presence of the 60 kDa Seladin-1 band in the rat adrenal gland, brain, and cerebellum. This band was not detected when Seladin-1 antiserum was replaced by the pre-immune serum at same dilution and exposure time or when the Seladin-1 antiserum was neutralized by the peptide raised against Seladin-1, indicating specificity of the antibody. A previous study had reported a Seladin-1 MW of 60 kDa in normal and Alzheimer’s disease brain tissues from 62- to 92-year-old patients (Greeve et al. 2000). The differences between these values may be related to differences in species (human versus rat) or age.
Figure 7 Immunofluorescence detection of Seladin-1 in 3-day zona fasciculata cultured cells. Impact of ACTH and U18666A (Seladin-1 inhibitor). The cells were cultured for 3-days in the absence (control, A, C) or in the presence of ACTH (10 nM) (B and D) and in the presence of 75 nM U18666A alone (E) or in combination with ACTH (F). After formaldehyde fixation and permeabilization with 0.1% Triton X-100, the cells were processed for immunofluorescence detection of Seladin-1 (1:1000) coupled to Alexa-488 (1:500, green), Golgi apparatus by the use of wheat germ agglutinin (WGA) lectin coupled to Alexa-594 (1:500, red) and nucleus (DAPI, blue). The resulting yellow color in merged images indicates colocalization of Seladin-1 with the WGA (Golgi apparatus) while the resulting light blue color indicates colocalization of Seladin-1 with DAPI (nucleus). All images were acquired with a ×100 objective. Scale bars, 5–6 μm (magnification: ×3500 for C, D and ×4500 for E, F).
There are currently a number of Seladin-1 mRNA sequences and predictive sequences that are now associated with a Seladin-1 protein larger than 60 kDa. For example, two recent predictive rat Seladin-1 sequences were shown to have a calculated MW of 86 kDa, hence clearly over 60 kDa.

At both the mRNA and the protein levels, Seladin-1 was observed to be more abundant in the zona fasciculata of control and ACTH-treated animals. This result is in agreement with previous studies conducted on human adrenal gland and human fasciculata cells in culture, at the mRNA level (Sarkar et al. 2001, Luciani et al. 2004). Moreover, the present results indicate that Seladin-1 localization is ubiquitous in the adrenal gland and that its presence is more important for adrenocortical than for adrenal medullar functions.

In control glomerulosa cells, Seladin-1 was mostly expressed in the membrane and nuclear fractions. Upon ACTH-treatment, the protein was greatly reduced in the cytosolic and membrane compartments, while conversely increasing in the cytoskeletal fraction. In fasciculata cells, the protein was localized mainly in nuclear, cytoskeletal, and membrane fractions. ACTH treatment reduced expression in the cytoplasm, with a concomitant relocalization into the nuclear fraction. These results suggest that the nuclear Seladin-1 function is more important in fasciculata than in glomerulosa cells. Through the use of WGA lectin as a marker of the Golgi apparatus, immunofluorescence studies demonstrate that, in the cytoplasm, the protein was mainly associated with the Golgi apparatus. Such localization has also been

![Graphs showing steroid secretion](image)

**Figure 8** Impact of Seladin-1 inhibitor, U18666A, on steroid secretion by zona glomerulosa cells (aldosterone) (A and B) and zona fasciculata cells (corticosterone) (C and D) cultured for 3 days. Steroids were measured by RIA as described in Materials and Methods. Secretion was measured after stimulation for 2 h (A and C) or after 3-day treatment (B and D) with ACTH (10 nM) or in the absence or in the presence of 25 and 75 nM U18666A. Results are expressed as means ± S.E.M. (n=4). Statistical significance when compared with respective control conditions: *P<0.05; †P<0.01, comparison between control U18666A groups of cells; ‡P<0.05; §P<0.01, comparison between ACTH-treated cells.
reported by Greeve et al. (2000) where, in a transfected human neuroglioma H4 cell line, Seladin-1—enhanced green fluorescent protein was localized in the endoplasmic reticulum and to the Golgi complex. Localization of Seladin-1 in Golgi may be correlated with a role in steroidogenesis since it is well known that in adrenocortical cells, the Golgi apparatus plays important functional and regulatory roles in corticosterone synthesis and that ACTH stimulation often leads to Golgi hypertrophy (Magalhaes et al. 1991, Cheng & Kowal 1997). These data may explain why a modification in Golgi structure is observed in cells stimulated with ACTH in the present model. This may suggest that the Seladin-1 localization to the Golgi apparatus is important for steroid secretion.

Furthermore, when glomerulosa and fasciculata cells in culture are stimulated with ACTH, there is a relocalization of Seladin-1 in specific nuclear regions. Wu et al. (2004) have recently described a massive translocation of Seladin-1 from the cytoplasm to the nucleus in Seladin-1-transfected cells exposed to 0.5 mM H2O2. The authors attributed a major role of Seladin-1 in the cellular response to oxidative stress, due to its ability to bind both the tumor suppressor p53 and the E3-ubiquitin-ligase Mdm2 and to displace Mdm2 from p53, thus protecting p53 from Mdm2-induced degradation and enabling p53 accumulation (Wu et al. 2004). It is well established that intense steroidogenesis in zona fasciculata leads to oxidative stress due to the occurrence of lipid peroxidation and the production of reactive oxygen species and toxic derivatives, such as isocaproaldehyde (Hornsby & Crivello 1983, Lefrancois-Martinez et al. 1999). This may explain the important quantity of endogenous anti-oxidant compounds (vitamin E, β-carotene, and vitamin C; Hornsby & Crivello 1983) and the presence of enzymes implicated in the endogenous detoxification of steroidogenesis by-products (Martinez et al. 2001) in the adrenal gland. Since Seladin-1 is an oxido-reductase enzyme (Mushegian & Koonin 1995), and in agreement with the results of Wu et al. (2004), the present results of a prominent Seladin-1 accumulation in specific nuclear regions may thus reflect an ACTH-induced regulation of Seladin-1 association with nuclear partners, such as p53 and Mdm2. The ACTH-induced Seladin-1 relocalization into specific nuclear regions is inhibited by U18666A suggesting an implication of the Δ24-reductase activity of Seladin-1. This hypothesis is supported by the observation that ACTH has a protective effect against oxidative stress (Pomeraniac et al. 2004). Together, the observation that ACTH induces relocalization in specific intranuclear compartments raises the hypothesis that Seladin-1 may play a major role in protecting adrenocortical cells against negative side effects due to intense steroidogenesis, either as a new major participant in the p53-Mdm2 interplay and/or through its own oxido-reductase activity. Such nuclear relocalization was not observed with Ang II treatment in glomerulosa cells, further supporting the notion that nuclear relocalization is associated with intense steroidogenesis. Indeed, Ang II stimulates aldosterone secretion by 2– to 5-fold, compared with the 30– to 60-fold increase observed with ACTH (Gallo-Payet & Payet 1989, Campbell et al. 2003).

The role of Seladin-1 in steroid secretion was assessed herein through the use of U18666A. At low concentrations (nM–μM), U18666A is a high-affinity binding inhibitor of the Δ24-reductase activity of Seladin-1, resulting in desmosterol accumulation (Bae & Paik 1997, Fliesler et al. 2000, Cenedella et al. 2004). In the present study, U18666A was used at concentrations ranging from 25 to 75 nM because, at this concentration, it does not induce adrenal cell toxicity and has little impact on cholesterol trafficking (Feng et al. 2003). Our results indicate that U18666A increases basal steroid secretion, but decreases ACTH-responsiveness for both aldosterone and corticosterone. The observation that U18666A effects on basal– and on ACTH-stimulated cells are more pronounced on aldosterone production than on corticosterone production may stem from a lower expression level of Seladin-1 in zona glomerulosa cells, resulting in less residual functional Seladin-1 following U18666A treatment.

Stimulation of basal secretion by this inhibitor may be due to desmosterol accumulation produced by U18666A-induced Seladin-1 inhibition. Desmosterol is a better substrate than cholesterol for steroidogenesis. It is more efficiently esterified than cholesterol, an essential step in sterol transport (Nordby & Norum 1975), and is fourfold more efficient as a substrate for the side chain cleavage reaction that produces precursors for steroidogenesis (Arthur et al. 1976, Mason et al. 1978). However, desmosterol has also been recently identified as a major negative regulator of cholesterol metabolism, being a strong inhibitor of sterol-regulatory element-binding protein 2 processing and a strong repressor of hydroxy-methyl-glutaryl-coenzyme A (HMG-CoA) reductase and low-density lipoprotein receptor expression (Yang et al. 2006). Therefore, by controlling desmosterol levels, the Δ24-reductase activity of Seladin-1 may play a major and dual role in regulating cholesterol uptake and biosynthesis, thereby modulating steroidogenesis. From these observations, low desmosterol concentrations may have positive effects on steroidogenesis, as a better substrate than cholesterol, which may explain the observed U18666A-induced increase in basal secretion. However, in ACTH-treated cells, the U18666A-induced desmosterol accumulation may impair the larger ACTH-induced increase in steroidogenic machinery. Seladin-1 may therefore act as a negative regulator of basal steroidogenesis when localized in the Golgi. Conversely, ACTH actions on Seladin-1 expression and subcellular localization may have two complementary effects: the first being the removal of the inhibition of secretion in the cytoplasm, whereas the second action would be associated with protection against ACTH-induced oxidative stress.

Seladin-1 expression has also been reported to be altered in adrenocortical adenomas and carcinomas, being overexpressed or repressed depending on tumor type (Sarkar et al. 2001, Luciani et al. 2004). However, Seladin-1 is also
abundantly expressed in mesenchymal stem cells (Benvenuti et al. 2006). On the other hand, U18666A induces apoptosis in cortical neurons in primary cultures (Koh & Cheung 2006). These observations suggest that Seladin-1 is involved in the balance between proliferation and apoptosis. However, such a role was not observed in glomerulosa cell cultures, since U18666A treatment had no effect on the high level of proliferation.

In summary, the results obtained in the present study are the first to assess Seladin-1 expression and distribution in the adrenal gland in vivo and in the rat primary cultures of adrenocortical cells. Using a multi–faceted approach, the study demonstrates that the protein expression of Seladin-1 is more abundant in fasciculata cells than in glomerulosa cells and that ACTH treatment increases both expression and nuclear localization of Seladin-1. Taken together, the results obtained herein, combined with previous data, suggest that depending on its cellular localization, Seladin-1 plays a major role in steroid secretion in the adrenal gland. It is proposed that the Δ24-reductase activity of Seladin-1 may be engaged in maintaining low levels of steroidogenesis. ACTH treatment induces a relocalization of Seladin-1 into the nucleus, thus facilitating ACTH-induced stimulation of steroidogenesis in the cytoplasm. Concomitantly, Seladin-1, in the nucleus, could be involved (through possible association with p53 and Mdm2 as partners), in protection against ACTH-induced oxidative stress, an undesirable consequence of intense steroidogenesis.

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