Rasd1 is an estrogen-responsive immediate early gene and modulates expression of late genes in rat anterior pituitary cells

Linhong Wang, Tetsuo Mitsui, Maho Ishida, Michi Izawa and Jun Arita

Department of Physiology, Graduate School of Medicine, University of Yamanashi, Chuo 409-3898, Japan

Abstract. Dexamethasone-induced Ras-related protein 1 (Rasd1) is a member of the Ras superfamily of monomeric G proteins that have a regulatory function in signal transduction. Here we investigated the role of Rasd1 in regulating estrogen-induced gene expression in primary cultures of rat anterior pituitary cells. Rasd1 mRNA expression in anterior pituitary cells decreased after treatment with forskolin or serum and increased after treatment with 17β-estradiol (E2). Increases in Rasd1 mRNA expression occurred as early as 0.5 h after E2 treatment, peaked at 1 h and were sustained for as long as 96 h. This rapid and profound increase in Rasd1 mRNA expression induced by E2 was also seen in GH4C1 cells, an estrogen receptor-positive somatolactotroph cell line. Among pituitary estrogen-responsive late genes studied, basal mRNA expression of Pim3 and Igf1 genes was decreased by RNA interference-mediated knockdown of Rasd1 expression, whereas basal expression of the Giot1 gene was increased. Moreover, Rasd1 knockdown enhanced stimulation of Pim3 mRNA expression and attenuated inhibition of Fosl1 mRNA expression 24 h after E2 treatment. These changes in mRNA expression were accompanied by enhanced activity of promoters containing CRE, AP-1 and SRE binding sequences. These results suggest that Rasd1 is an estrogen-responsive immediate early gene and modulates E2 induction of at least several late genes in anterior pituitary cells.

Key words: Estrogen, Rasd1, Pituitary, Lactotroph, Estrogen-responsive gene

Estrogens stimulate the normal growth and differentiation of several estrogen-responsive organs including the uterus, mammary gland and anterior pituitary gland and are also involved in the pathogenesis of tumors in these organs [1]. Estrogen action is mediated by the estrogen receptor (ER), which regulates target gene expression as a ligand-activated transcription factor. Nuclear ER-α and -β directly bind to estrogen response elements (EREs), specific DNA sequences in the regulatory regions of target genes, and then activate transcription through the recruitment of coactivator proteins [2]. Alternatively, ERs bind to other transcription factors such as AP-1, Sp1 and NFκB proteins and indirectly modulate gene expression through protein-protein interactions [3]. Furthermore, recent studies revealed that ERs in the cytoplasmic membrane could have non-genomic actions mediated through protein kinase activation [4].

The anterior pituitary gland contains ER-positive cells [5], and pituitary lactotrophs are typical estrogen-responsive cells that can be stimulated to proliferate by estrogen exposure both in vivo and in vitro [6-8]. We previously performed global gene expression profiling using anterior pituitary cells in primary culture and identified a number of estrogen-responsive genes [9]. In particular, dexamethasone-induced Ras-related protein 1 (Rasd1) showed up-regulated expression in response to 17β-estradiol (E2).

Rasd1 is a member of the Ras family of monomeric G proteins that play central roles in multiple cellular functions, including cell proliferation, differentiation, transformation, secretion and apoptosis [10]. Interactions between Rasd1 and G10/Goα modulate heterotrimeric G-protein signaling pathways independently of a G-protein-coupled receptor. Rasd1 inhibits adenylyl cyclase through Gαi, leading to inhibition of cAMP response element-binding protein (CREB) activation [11], and blocks dopamine receptor-mediated heterologous sensitization of adenylyl cyclase 1 via Gβγ [12]. Rasd1 also forms an apparent ternary complex with...
neuronal nitric oxide synthase (nNOS) and the nNOS-binding protein CAPON, which directs and enhances the delivery and specificity of nitric oxide signals in the nervous system [13, 14]. Rasd1 expression was shown to inhibit cell growth of NIH-3T3 fibroblast cells, the MCF-7 breast cancer cell line and the A549 lung adenocarcinoma cell line [15].

Although Rasd1 expression is induced in response to glucocorticoids in AtT-20 cells, a pituitary corticotroph cell line [16], the role of Rasd1 in the anterior pituitary gland remains largely unknown. In the present study, we investigated the regulation of Rasd1 mRNA expression in rat pituitary cells in primary culture and found that Rasd1 was an estrogen-responsive immediate early gene that showed increased expression as early as 0.5 h after E2 treatment. We also determined the effects of knockdown of Rasd1 mRNA on E2 induction of late genes.

Materials and Methods

Cell culture

Seven-week-old female Wistar rats, purchased from Japan SLC (Shizuoka, Japan), were used to obtain anterior pituitary cells for the primary cultures. All experiments involving animals were approved by the Ethical Committee of Animal Experiments of the University of Yamanashi. Anterior pituitary cells were dispersed as described previously [17]. A 200-300 μL aliquot of a cell suspension containing 4-6×10⁵ pituitary cells was plated on poly-D-lysine-coated 35 mm culture dishes. For adenovirus infection experiments, adenovirus vectors were added to the cell suspensions at appropriate multiplicities of infection (MOIs) when the pituitary cells were plated. The cells were allowed to attach to the surface of the dishes in a humidified CO₂ incubator for 1 h and then flooded with 2 mL of a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s Nutrient Mix F-12 without phenol red and containing 15 mM HEPES, penicillin and streptomycin (DMEM/F12) supplemented with 500 ng/mL insulin and cultured at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air for 1 day. The pituitary cells were then cultured with the same restriction enzymes in the Adeno-X Expression System according to the manufacturer’s protocol (Clontech Laboratories, Mountain View, CA, USA). The oligonucleotide sequences for Rasd1-targeted and control short hairpin RNAs (shRNAs) were designed with the siRNA Design Support View, CA, USA). The oligonucleotide sequences for Adenovirus vectors

Adenovirus vectors were produced using the Adeno-X Expression System according to the manufacturer’s protocol (Clontech Laboratories, Mountain View, CA, USA). The oligonucleotide sequences for Rasd1-targeted and control short hairpin RNAs (shRNAs) were designed with the siRNA Design Support System (Takara Bio, Otsu, Japan). The oligonucleotides synthesized for rat Rasd1 shRNA were 5'-GA TCCGAAAAGAACTAGCTAAGACCTGTAAGC CACAGATGGGAGTCTTAGCTAGTTCTTTC TTTG-3' and 5'-ATTCAAAAAGAAAGAACTA CTAAAGACTCCCATCTGTGGCTTCACAGAGT CTAGCTAGTTCTTCG-3’, and those synthesized for control rat shRNA were 5'-GATCCGCCGTC CGCATTAGTACCGGGCTGTGAAGCCACAGAT GGCCTCGCTAATGCGGAACGGTTTTGG-3' and 5'-AATTCAAAAAGCGCTCGCCATAGTACGG GCCCATCTGTGGCTTCACACCGCTACTA ATGCGGACGGCG-3’ (underlined letters indicate sequences targeted for RNA interference). The oligonucleotides were annealed and ligated downstream of the human U6 promoter in the pSIREN-Shuttle vector (Clontech). The pSIREN-Shuttle vectors containing the oligonucleotides were digested with I-CeuI and P1-SceI, and the genes were inserted into a site created with the same restriction enzymes in the Adeno-X viral DNA as described previously [9]. Recombinant adenoviruses were generated from the Adeno-X viral DNA and purified as described previously [20]. Adenoviruses expressing shRNA Rasd1 and control short hairpin sequences are referred to as Ad-shRasd1 and Ad-shCont, respectively.

Ad-CRE/Luc and Ad-SRE/Luc are adenovirus
Vectors carrying a firefly luciferase reporter gene driven by a TATA-like promoter (P_{Tal}) containing the consensus cAMP response element (CRE) and serum response element (SRE) sequences, respectively, as described previously [20]. Ad-NFκB/Luc is an adenovirus vector carrying a firefly luciferase reporter gene driven by the P_{Tal} promoter containing the consensus NFκB protein binding sequence as described previously [21]. Ad-AP-1/Luc, an adenovirus vector carrying a firefly luciferase reporter gene driven by the P_{Tal} promoter containing the consensus activator protein-1 (AP-1)-binding sequence was produced from the pAP1-Luc vector (Mercury Pathway Profiling System, Clontech) in a manner similar to that for Ad-CRE/Luc and Ad-SRE/Luc. Ad-Tal/hrLuc, an adenovirus vector carrying a Renilla luciferase reporter gene driven by the P_{Tal} promoter lacking any consensus sequence, was produced as described previously [22].

Quantitative real-time PCR (qRT-PCR)

Expression levels of mRNAs for estrogen-responsive genes were determined by qRT-PCR as described previously [23]. Total pituitary RNA was isolated using an RNasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol and treated with RNase-free DNase I (Qiagen). RNA (0.5 µg) was reverse-transcribed using oligo(dT)20 primer and ReverTra Ace® reverse transcriptase kit (Toyobo, Osaka, Japan). Reverse transcriptase reactions were amplified using an ABI prism 7,500 Sequence Detection System (Applied Biosystems, Forster City, CA, USA) with SYBR Green Real-Time PCR Master Mix (Toyobo). The gene-specific primers were designed using Primer Express software (Applied Biosystems). The sequences of the primers used were: FBJ osteosarcoma oncogene (Fos), 5′-AGCAGGCGAGGCGAAA -3′ and 5′-AGCTCTCTCCTCGATTCG GG-3′, Jun proto-oncogene, AP-1 transcription factor subunit (Jun), 5′-CTTCTGGCGGCTGCTGAG-3′ and 5′-ACTGAGCTAGGCAGCCAC-3′, myelocytomatosis oncogene (Myec), 5′-ATGAGCTCTCAAGGC TAAACGC-3′ and 5′-GGCAGTTACATATGCTGG AAGTC-3′. Primers specific for the estrogen-responsive gene ATP-binding cassette, subfamily G (WHITE), member 2 (Abcg2), basic leucine zipper ATF-like transcription factor 3 (Batf3), cyclin D1 (Cndd1), FOS like 1, AP-1 transcription factor subunit (Fosl1), gonadotropin inducible ovarian transcription factor 1 (Giot1), insulin-like growth factor 1 (Igf1), neuronal pentraxin 1 (Nptx1), PDZ and LIM domain 3 (Pdlim3), Pim-3 proto-oncogene, serine/threonine kinase (Pim3), Rasd1, transcription factor CP2-like 1 (Tcfcpl21) and wingless-type MMTV integration site family, member 4 (Wnt4) were described previously [9,24]. Thermal cycling was conducted at 95 °C for 60 s, followed by 40 cycles of amplification at 95 °C for 15 s and 60 °C for 45 s. The thermal amplification was followed by a dissociation-curve analysis to confirm the specificity of the amplification products. The mRNA levels were calculated using the 2^{ΔΔCT} method with acid ribosomal phosphoprotein P0 (Arbp) as an endogenous reference.

Promoter activity assays

Cells were lysed with 400 µL Reporter Lysis Buffer or 200 µL Renilla Luciferase Assay Lysis Buffer according to the manufacturer’s protocol. Luciferase assays were performed with the Luciferase Assay System and Renilla Luciferase Assay System (Promega, Madison, WI, USA). The promoter activities derived from the luciferase assays were normalized relative to those derived from Renilla luciferase assays.

Statistical analysis

Experiments were carried out using three culture dishes per experimental group and replicated three times with separate batches of cell preparations unless otherwise stated. Differences between groups were statistically analyzed using Student’s t-test or one-way ANOVA followed by Fisher’s protected least significant difference test.

Results

Rasd1 is an estrogen-responsive immediate early gene in anterior pituitary cells

We first determined whether Rasd1 mRNA expression levels in primary cultures of anterior pituitary cells were affected by various agents such as IGF-1, the cAMP-increasing agent forskolin and serum, as well as E2. Pituitary cells were treated with 30 ng/mL IGF-1, 1 µM forskolin, 10% DCC-HS or 1 nM E2 for 24 h, and the amount of Rasd1 mRNA was determined by qRT-PCR. Rasd1 mRNA levels were not altered by IGF-1 treatment, but significantly decreased following treatment with forskolin or DCC-HS (p<0.05) (Fig. 1).
In contrast, \textit{Rasd1} mRNA levels were increased 3.5-fold by treatment with E2 ($p<0.05$).

To determine the time course of \textit{Rasd1} mRNA expression following E2 treatment, pituitary cells were treated with E2 for 0.5, 1, 2, 24 and 96 h. \textit{Rasd1} mRNA expression levels exhibited a rapid increase in response to E2 within 0.5 h and peaked at 1 h with a 4.6-fold increase over vehicle-treated cells (Fig. 2, \textit{left panel}). The rapid increase was reduced to approximately 2-fold above that of the vehicle by 2 h, and the lower levels were sustained thereafter up to 96 h.

To determine which cell type among anterior pituitary cells is responsible for E2 induction of \textit{Rasd1} mRNA expression, we examined E2-induced changes in \textit{Rasd1} mRNA in GH4C1 cells, an ER-positive somatolactotroph cell line. In GH4C1 cells, \textit{Rasd1} mRNA levels were increased 4.6-fold as early as 0.5 h after 10 nM E2 treatment, peaked at 1 h and then were reduced thereafter (Fig. 2, \textit{right panel}).

Next, we compared the time course of \textit{Rasd1} mRNA
expression levels with that of mRNA expression levels of other estrogen-responsive genes in GH4C1 cells. Based on the time course pattern, four classes of E2-induced changes were found. A rapid and profound increase in mRNA expression of the Myc gene was also observed at 0.5 h (Fig. 3 first row). The time course for the E2 responsiveness of Rasd1 and Myc genes was distinct from that of the other three classes: i) Pdlim3, Batf3 and Ccnd1 genes showed an mRNA expression peak 4 h after E2 treatment (second row); ii) Fos and Abcg2 genes had maximum mRNA expression levels at 24 h (third row); and iii) Jun, Giot1 and Tecfp2l1 gene expression had a negative response (fourth row).

**Rasd1 modulates the expression of pituitary estrogen-responsive late genes**

To examine whether Rasd1 acting as an estrogen-responsive immediate early gene modulates induction of other estrogen-responsive late genes, we generated Ad-shRasd1, an adenovirus vector expressing interfering shRNA directed toward Rasd1 mRNA. Ad-shRasd1 was effective in suppressing Rasd1 mRNA levels in adenovirus vector-infected pituitary cells in an infection dose-dependent manner in that 38% and 58% suppression was achieved at 2 and 4 MOIs, respectively, relative to cells infected with Ad-shCont, an adenovirus vector expressing shRNA for a negative control sequence (Fig. 4; p<0.05).

We investigated the effects of Rasd1 knockdown on basal and E2-induced mRNA expression of selected estrogen-responsive late genes in pituitary cells: the estrogen-up-regulated genes Abcg2, Batf3, Pdlim3, Pim3 and Wnt4, and the estrogen-down-regulated genes Ccnd1, Fosl1, Giot1, Igf1 and Nptx1. RNA interference-mediated knockdown of Rasd1 significantly decreased basal mRNA expression levels of Pim3 and Igf1 genes by 0.8-fold and 0.6-fold, respectively, and increased basal mRNA levels of the Giot1 gene by 1.7-fold compared to that seen with negative control knockdown (p<0.05) (Fig. 5A). The expected stimulation and inhibition of mRNA expression levels

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**Fig. 3** Time course of mRNA expression of Rasd1 and other estrogen-responsive genes after E2 treatment of GH4C1 cells

The genes in each row compose a distinct class in terms of mRNA expression. GH4C1 cells were cultured in DMEM/F12-CD containing 5% DCC-HS for 3 days and treated with vehicle or 10 nM E2 for 0.5, 4 and 24 h before the end of culture. The amounts of estrogen-responsive gene mRNA were determined by qRT-PCR analysis. mRNA levels are expressed as the fold-change relative to the vehicle-treated groups at the corresponding times. Data are presented as the mean ± SEM from a representative experiment.

**Fig. 4** Verification of RNA interference-mediated knockdown of Rasd1 mRNA expression

Pituitary cells were infected with Ad-shCont or Ad-shRasd1 at 2 or 4 MOIs for 3 days. Total RNA was collected, and mRNA levels were determined by qRT-PCR analysis. Rasd1 mRNA levels are expressed as the fold-change relative to the Ad-shCont-infected groups. Data are presented as means ± SEM of triplicate determinations from a representative experiment.

*Significantly different from the Ad-shCont-infected group (p<0.05).
of these estrogen-responsive genes occurred 24 h after treatment with 1 nM E2 in control knockdown pituitary cells (Fig. 5B). Out of ten genes, Rasd1 knockdown modified E2-induced mRNA levels wherein Pim3 mRNA expression was significantly enhanced by 1.5-fold, and E2 inhibition of Fosl1 mRNA expression was almost eliminated (p<0.05).

To determine whether modified expression of these estrogen-responsive late genes was accompanied by altered activities of promoters containing specific transcription factor binding sequences, we performed promoter activity assays for CRE, AP-1, SRE and NFκB binding sequences in pituitary cells infected with an adenovirus vector expressing reporter gene and that expressing either control shRNA or Rasd1-specific shRNA. Rasd1 knockdown significantly increased CRE promoter activity by 1.9-fold compared with control knockdown (p<0.05) (Fig. 6). AP-1 and SRE promoter activities were also significantly increased, but to a lesser degree than CRE (p<0.05), whereas NFκB promoter activity was not affected by Rasd1 knockdown.

**Discussion**

Rasd1 mRNA is expressed in many tissues throughout the body, including the heart, liver, kidney, lung, brain and particularly the anterior pituitary gland [13, 16, 25]. However, little is known about the regulation of Rasd1 expression in these tissues. To date, the only factors known to regulate Rasd1 expression are glucocorticoids [16, 25, 26] and estrogens [9, 26]. Therefore, in this study we determined whether other agents, especially mitogens that affect pituitary cell proliferation, affect Rasd1 mRNA expression. We showed that

![Fig. 5](image-url)
mRNA expression levels of Rasd1 in pituitary cells were not altered by the potent mitogen IGF-1, but were suppressed by serum supplementation and treatment with forskolin, which increases cAMP levels. The inhibitory effect of forskolin is noteworthy because others demonstrated that Rasd1 expression suppressed the adenyl cyclase/cAMP/protein kinase A/CREB pathway [11, 27, 28]. Taken together, these findings suggest the existence of a regulatory loop in which stimulation of adenyl cyclase/cAMP enhances its own activity and production by inhibiting Rasd1 expression.

Estrogen is effective in stimulating Rasd1 mRNA expression in vivo in mouse pituitary tissues [26]. Our previous study using DNA microarray analysis and qRT-PCR demonstrated that E2 stimulates Rasd1 mRNA expression in primary cultures of rat pituitary cells [9]. The results of the present study showed that E2 treatment stimulated Rasd1 mRNA expression in primary cultures of pituitary cells as early as 0.5 h after treatment. Among estrogen-responsive genes, Rasd1 appears to be an estrogen-responsive immediate early gene that has an expression time course that is similar to that of Myc [29]. In contrast, we found that expression of Fos and Jun genes, which in rat uterus act as immediate early genes [30, 31], did not change within 0.5 h of E2 treatment. The mechanism of ER regulation of Rasd1 expression requires further investigation to determine whether genomic actions mediated by direct binding to EREs or sequences recognized by other transcription factors such as AP-1, Sp1 and NfxB are involved. No ERE has been reported in the promoter region of the rat Rasd1 gene. As such, additional promoter analyses and characterization of ER-binding sites are needed to clarify the mechanism responsible for the rapid induction of Rasd1 gene expression.

Rasd1 is a new Ras superfamily gene that is expressed in response to dexamethasone treatment of the mouse corticotroph cell line AtT-20 [16]. Estrogen did not stimulate Rasd1 mRNA expression in AtT-20 cells, but did stimulate Rasd1 mRNA expression in vivo in mouse pituitary tissue as well as dexamethasone [26]. These results suggest that Rasd1 mRNA expressed in pituitary cell types other than corticotrophs is responsive to estrogen. Because lactotrophs are the primary cell type in the rat anterior pituitary [32, 33] and have ERs [5], this cell type may be responsible for the estrogen-responsiveness of Rasd1 expression. To test this possibility, we determined E2 responsiveness of Rasd1 mRNA expression in GH4C1 cells, an ER-positive somatolactotroph cell line. The present study demonstrates that E2 rapidly and profoundly increases Rasd1 mRNA expression in GH4C1 cells as well as anterior pituitary cells, suggesting that estrogen-induced Rasd1 expression occurs in pituitary lactotrophs. This is the first report identifying the cell type that has estrogen-responsive Rasd1 expression.

The finding that the Rasd1 gene is an estrogen-responsive immediate early gene raises the question of whether Rasd1 modulates expression of other pituitary genes that are induced by E2 later than that of Rasd1. To address this question, we investigated the effects of Rasd1 knockdown on mRNA induction of estrogen-responsive genes 24 h after E2 treatment. In addition to the changes of basal mRNA expression of Pim3, Igf1 and Giot1 genes by Rasd1 knockdown, we found notable changes in E2 late induction of Pim3 and Fosl1 genes that encode a protooncogene with serine/threonine kinase activity [34] and a component of the AP-1 transcription factor complex [35], respectively; E2 stimulation of Pim3 expression was enhanced and inhibition of Fosl1 expression was eliminated. These
results suggest that E2-induced inhibition of Fosl1 expression in pituitary cells is indirect and mediated by Rasd1 expression. The finding that out of ten genes selected, two genes, Fosl1 and Pim3, were influenced by Rasd1 knockout indicates that E2 induction of a considerable number of late genes in pituitary cells is modulated by or dependent on Rasd1 expression and suggests for the first time that the estrogen-responsive immediate early gene Rasd1 acts as a master gene for E2 induction of late genes.

In the present study we demonstrated that knockdown of Rasd1 mRNA expression markedly increased CRE-containing promoter activity, consistent with the finding that Rasd1 inhibits adenylyl cyclase leading to inhibition of CREB activation [11, 27, 28]. However, whether a change in CRE-containing promoter activity or a combination of changes in CRE, AP-1 and SRE promoter activities shown to be accompanied by Rasd1 knockdown indicates that E2 induction of a considerable number of late genes in pituitary cells is indirect and mediated by or dependent on Rasd1 expression and suggests for the first time that the estrogen-responsive immediate early gene Rasd1 acts as a master gene for E2 induction of late genes.

In conclusion, Rasd1 is unusual among Ras GTPases in that its expression is rapidly induced by E2. We identified pituitary lactotrophs as the cells in which E2 induces Rasd1 mRNA expression. Rasd1 is an immediate early gene that modulates E2-induced expression of estrogen-responsive late genes in pituitary cells. Thus, the rapid estrogen induction of Rasd1 mRNA expression may play an essential role in estrogen-regulated functions of anterior pituitary cells, in particular lactotrophs, by orchestrating the expression of estrogen-responsive late genes.

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Disclosure

None of the authors have any potential conflicts or interest associated with this research.

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