Up-regulation of DUSP5 and DUSP6 by gonadotropin-releasing hormone in cultured hypothalamic neurons, GT1-7 cells

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) is secreted from hypothalamic neurons (GnRH neurons) and stimulates anterior pituitary gonadotrophs to synthesize and secrete gonadotropins. In addition to gonadotrophs, GnRH neurons also express GnRH receptors, and the autocrine action of GnRH is reportedly involved in the regulation of functions of GnRH neurons. There is accumulating evidence that extracellular signal-regulated kinase (ERK), one of mitogen-activated protein kinases (MAPKs), is activated by GnRH and involved in various effects of GnRH in GnRH neurons. In the present study, we performed microarray analysis to examine the types of genes whose expression was regulated by GnRH in immortalized mouse GnRH neurons (GT1-7 cells). We found that 257 genes among 55,681 genes examined were up-regulated after 30-min treatment of GT1-7 cells with GnRH. These up-regulated genes included four dual-specificity MAPK phosphatases (DUSPs), DUSP1, DUSP2, DUSP5, and DUSP6. Reverse transcription-polymerase chain reaction analysis confirmed that the mRNA levels of DUSP5 and DUSP6 were robustly increased within 30 min. U0126, an inhibitor of ERK activation, completely inhibited the increases in the mRNA levels of DUSP5 and DUSP6. Immunoblotting analysis revealed that ERK activation peaked at 5 min and declined steeply at 60 min, whereas DUSP5 and DUSP6 proteins were increased from 60 min. It was notable that down-regulation of DUSP6 augmented GnRH-induced ERK activation approximately 1.7-fold at 60 min. These results suggested that the up-regulation of DUSP6 regulates the duration of ERK activation at least in part.

Gonadotropin-releasing hormone (GnRH) is secreted from hypothalamic neurons (GnRH neurons) and stimulates anterior pituitary gonadotrophs to synthesize and secrete two gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The gonadotropins stimulate spermatogenesis, folliculogenesis, and ovulation; therefore, GnRH is the first key hormone of reproduction (for review, see Ref. 13). The GnRH receptor belongs to a class of

G-protein-coupled receptors that activate phospholipase C β (13). In addition to gonadotrophs, GnRH neurons also express GnRH receptors, and the autocrine action of GnRH is reportedly involved in the regulation of functions of GnRH neurons (for review, see Ref. 9).

Because the mammalian brain contains only 800–1,000 GnRH neurons, it is necessary to use immortalized GnRH neurons in studies of signal transduction after stimulation of the GnRH receptor. GT1-7 cells, immortalized GnRH neurons, were generated by genetically targeted tumorigenesis in transgenic mice (12). GT1-7 cells retain many of the characteristics of native GnRH neurons, including the expression of GnRH receptors (12). GnRH treatment of GT1-7 cells activates mitogen-activated protein kinases

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(MAPKs), including extracellular signal-regulated kinase (ERK) (13). MAPKs are activated through phosphorylation at both threonine and tyrosine residues within the activation loop, and dephosphorylation of the threonine and/or tyrosine residue results in complete inactivation of MAPKs (for review, see Ref. 7). In our previous study, we found that the activation of ERK after GnRH treatment of GT1-7 cells peaked after 5–15 min and then declined steeply (16). These results suggested that protein phosphatases rapidly dephosphorylated and inactivated ERK after GnRH treatment.

Accumulating evidence indicates that dephosphorylation of MAPKs is conducted by serine/threonine phosphatases, tyrosine phosphatases, and dual specificity MAPK phosphatases (DUSPs). Among these protein phosphatases, DUSPs are unique because they have a MAPK-binding (MKB) domain in the N-terminal half (3). When MAPKs bind to the MKB domain, the DUSP domain in the C-terminal half becomes activated to dephosphorylate the bound MAPK. These properties indicate that DUSPs specifically dephosphorylate and inactivate MAPKs. It has been reported that GnRH treatment of cultured gonadotrophs induced DUSP1 and DUSP2 (14, 17). However, the up-regulation and the physiological roles of DUSPs have not been reported in GT1-7 cells.

In the present study, we performed microarray analysis to examine the types of genes whose expression was regulated by GnRH in GT1-7 cells. The analysis suggested that the genes for four types of DUSPs, DUSP1, DUSP2, DUSP5, and DUSP6, were up-regulated after GnRH treatment. Reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed that the mRNA levels of DUSP5 and DUSP6 were robustly increased within 30 min. In addition, we confirmed that both DUSP5 and DUSP6 were increased at the protein levels within 1 h. Furthermore, we examined the possibility that the negative feedback mechanisms might exist for GnRH-induced ERK activation in GT1-7 cells.

MATERIALS AND METHODS

Materials. The following chemicals and reagents were obtained from the indicated sources: fetal calf serum, HyClone (Logan, UT, USA); [des-Gly¹⁰], [D-Ala⁶]-LH-RH Ethylamide (GnRH), poly-L-lysine, anti-ERK antibody (M5670), and phosphate-buffered saline (PBS), Sigma Chemical Co. (St Louis, MO, USA); anti-active ERK antibody (V8031), Promega Corp. (Madison, WI, USA); anti-DUSP6 antibody

(rabbit monoclonal antibody, clone number EPR129Y), Abcam (Cambridge, UK); anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody, GeneTex (Irvine, CA, USA); DynaMarker Protein MultiColor, BioDynamics Lab. (Tokyo, Japan); Dulbecco's modified Eagle's medium, Nissui Pharmaceutical Co. (Tokyo, Japan); anti-DUSP5 antibody (PAB2152) and HL-60 cell lysate, Abnova Co. (Taipei, Taiwan); anti-DUSP5 antibody (H-74), Santa Cruz (Santa Cruz, CA); sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards, Bio-Rad (Richmond, CA, USA); Other chemicals were of analytical grade.

Cell culture. GT1-7 cells were kindly provided by Dr. R. Weiner (University of California) and Dr. M. Kawahara (Musashino University) (8, 12). The cells were grown on 0.02% (wt/vol) poly-L-lysine-coated Petri dishes (NIPPON Genetics, Tokyo, Japan) as described previously (16). GT1-7 cells were treated with or without U0126 and GnRH as indicated. We chose the concentration of U0126 as directed by the manufacturer's instructions.

Microarray analysis and reverse transcription-polymerase chain reaction. After treatment of GT1-7 cells with 50 nM GnRH for 30 min, total RNA was extracted using an RNeasy Mini Kit (Qiagen, MD) in accordance with the manufacturer's directions, and the amount of total RNA was determined by measuring absorbance at 260 nm using a Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA). The expression profile of mRNA was determined using a SurePrint G3 mouse GE Microarray (8 × 60K v2) (Agilent Technology). The experiment was performed by Cell Innovator Inc. (Fukuoka, Japan). The genes for which the Z score and the ratio were above 2.0 and 1.5, respectively, were considered up-regulated. In contrast, the genes for which the Z score and the ratio were below −2.0 and 0.66, respectively, were considered down-regulated. All microarray data were MIAME compliant, and the raw data have been deposited in the Gene Expression Omnibus (GEO) database, with the accession number GSE112857.

For RT-PCR, first-strand cDNA was synthesized from 2 µg of total RNA in a 20 µL reaction volume using AMV Reverse Transcriptase (Promega Co., Madison, WI) with an Oligo dT-Adaptor primer. The oligonucleotide sequences of the primers are shown in Table 1. Preliminary PCR experiments were performed to identify the linear amplification conditions for each product. Amplification of DUSP1, DUSP2,

Table 1 Oligonucleotide sequence of PCR primers and PCR Product Size

DUSP	Oligonucleotide sequence	Product size (bp)
DUSP1	5'-CTCATGGGAGCTGGTCCTTA-3' Sense 5'-GCGAAGAACTGCCTCAAAC-3' Antisense	214
DUSP2	5'-CAAGGGCTCAGAAAACAAGC-3' Sense 5'-CAGCACCAATTACAGCGAGA-3' Antisense	185
DUSP5	5'-TGCACCACCCACCTACACTA-3' Sense 5'-CATGAGGTAAGCCATGCAGA-3' Antisense	183
DUSP6	5'-TTGAATGTCACCCCCAATT-3' Sense 5'-CATCGTTCATGGACAGGTTG-3' Antisense	247
GAPDH	5'-ACCACAGTCCATGCCATCAC-3' Sense 5'-TCCACCACCCTGTTGCTGTA-3' Antisense	443

DUSP5, DUSP6, and GAPDH was performed for 36, 37, 38, 37, and 32 cycles (30 s at 94°C, 1 min at 58°C, 1 min at 70°C), respectively, with Expand Long-Range dNTPack (Roche Diagnosis, Indianapolis, IN). The PCR products were separated by electrophoresis in a 1.5% agarose gel, visualized by ethidium bromide staining, and quantified using an ImageQuant LAS 4000 mini (GE Healthcare UK Ltd., Little Chalfont, UK) with Multi Gauge software (version 3.1).

Preparation of cell extract. GT1-7 cells were lysed in 1 × SDS-PAGE sample buffer containing 2% (wt/vol) SDS, 62.5 mM Tris-HCl (pH 6.8), 5% (vol/vol) 2-mercaptoethanol, 5% (vol/vol) glycerol, and 0.01% (wt/vol) bromophenol blue (4, 10). The cell lysate was sonicated for 10 s at room temperature, and heated to 98°C for 5 min to use as a cell extract. The cell extract was kept at -80°C until use.

Production of the antibody against DUSP5. A DUSP5 peptide (CLDGRQLRKMRLKEA), corresponding to the N-terminal amino acids of mouse and human DUSP5, was synthesized by Scrum Inc. (Tokyo, Japan). The cysteine residue in this DUSP5 peptide was coupled to hemocyanin from a keyhole limpet, and antiserum were obtained by Scrum Inc. by immunizing two rabbits five times at intervals of 2 weeks. The IgG fraction from one rabbit was prepared from the antiserum by ammonium sulfate fractionation (0–50%), as described previously (11). Immobilization of the DUSP5 peptide on a 2 mL SulfoLink column and affinity purification of the antibody were performed in accordance with the manufacturer's instructions using a SulfoLink Immobilization Kit for Peptides (Pierce Chemical Co., Rockford, IL). We applied the IgG fraction to the DUSP5 peptide-immobilized column, and the antibody was eluted from the column using 0.1 M gly-

cine (pH 3.0). To the fractions, Tris-HCl (pH 8.0) and bovine serum albumin were added to final concentrations of 95 mM and 1 mg/mL, respectively, and subjected to an enzyme-linked immunosorbent assay (ELISA). The purified antibody (anti-DUSP5 antibody) was kept in aliquots at -80°C.

SDS-PAGE and immunoblotting analysis. SDS-PAGE was performed by the method of Laemmli (10), and immunoblotting analysis was performed as described previously (4, 15). Immunoreactive proteins were detected using an enhanced chemiluminescence detection kit (GE Healthcare UK Ltd.) as directed by the manufacturer. We quantified immunoreactivity using an ImageQuant LAS4000 mini (GE Healthcare UK Ltd.) with Multi Gauge software (version 3.1). For reprobing, the membrane was incubated with stripping buffer containing 62.5 mM Tris-HCl (pH 6.7), 100 mM 2-mercaptoethanol, and 2% (wt/vol) SDS at 50°C for 30 min (16). The membrane was then washed with a blocking solution containing 5% (wt/vol) skim milk, 100 mM Tris-HCl (pH 7.5), 0.9% (wt/vol) NaCl, and 0.1% (vol/vol) Tween-20 followed by Tris-buffered saline with Tween-20 containing 100 mM Tris-HCl (pH 7.5), 0.9% (wt/vol) NaCl, and 0.1% (vol/vol) Tween-20 at room temperature and subjected to immunoblotting analysis.

siRNA transfection. Small interfering RNAs (siRNAs) against mouse DUSP5 and DUSP6, and control siRNA, were obtained from Qiagen (Valencia, CA, USA). The siRNAs used were as follows: DUSP5, 5'-CCACCCACCUACACUACAATT-3'; and DUSP6, 5'-CCAUGAUGUUCGUGGUGUATT-3'. siRNA was introduced into GT1-7 cells using a Neon Transfection System Kit (Invitrogen, Carlsbad, CA, USA) as directed by the manufacturer. siRNA at 40 nM was transfected into 0.5–1 × 10⁶ cells per 35-mm

Table 2 30 genes and DUSP2 that were up-regulated by GnRH

Number	Gene Symbol	Gene Title	Z score	Ratio
1	Npas4	Neuronal PAS domain protein 4	43.26	55.22
2	Egr1	Early growth response 1	36.72	352.67
3	Fosb	FBJ osteosarcoma oncogene B	27.65	82.78
4	Egr2	Early growth response 2	27.17	270.13
5	Fos	FBJ osteosarcoma oncogene	26.01	63.69
6	Nr4a1	Nuclear receptor subfamily 4, group A, member 1	22.06	4.79
7	Htr2a	5-hydroxytryptamine (serotonin) receptor 2A	20.33	65.99
8	Foxr1	Forkhead box R1	19.32	53.58
9	Ccni	Cyclin I	18.03	3.17
10	Jun	Jun oncogene	14.53	3.84
11	Ier2	Immediate early response 2	13.37	8.47
12	Arc	Activity regulated cytoskeletal-associated protein	12.88	7.83
13	Gdf15	Growth differentiation factor 15	11.84	3.00
14	Srf	Serum response factor	11.71	2.96
15	Ubc	Ubiquitin C	11.44	1.60
16	Cyr61	Cysteine rich protein 61	11.26	6.04
17	Sult1a1	Sulfotransferase family 1A	11.17	160.99
18	Junb	Jun-B oncogene	11.08	9.81
19	Tbx19	T-box 19	10.70	129.97
20	Vmn1r40	Vomerolateral 1 receptor 40	10.39	113.05
21	C1ra	Complement component 1, r subcomponent A	10.23	105.02
22	Btg2	B cell translocation gene 2, anti-proliferative	9.92	4.88
23	Ccdc171	Coiled-coil domain containing 171	9.65	80.78
24	<u>Dusp5</u>	<u>Dual specificity phosphatase 5</u>	9.25	6.73
25	Olfir1095	Olfactory receptor 1095	8.85	56.16
26	Gem	GTP binding protein	8.61	2.22
27	Tceal7	Transcription elongation factor A (SII)-like 7	8.61	50.24
28	Postn	Periostin, osteoblast specific factor (Postn)	8.60	50.15
29	<u>Dusp6</u>	<u>Dual specificity phosphatase 6</u>	8.30	3.77
30	<u>Dusp1</u>	<u>Dual specificity phosphatase 1</u>	7.73	3.44
78	<u>Dusp2</u>	<u>Dual specificity phosphatase 2</u>	3.54	1.76

dish (5). The cells were kept at 37°C in a CO₂ incubator for 48 h.

Other procedures. Protein concentrations were determined using a Qubit Protein Assay kit with a Qubit 2.0 Fluorometer (Invitrogen). In pilot experiments, the protein concentration curves were linear in the presence of 0.04% (wt/vol) SDS, 0.1% (vol/vol) 2-mercaptoethanol, and 0.1% (vol/vol) glycerol. Therefore, we diluted the cell extract 50-fold with water for protein quantification. We repeated the experiments at least three times with reproducible results, and representative results are shown in the figures. The values are expressed as the mean $\pm$ SE. Statistical analysis was performed using a one-way ANOVA plus Duncan's multiple range test. $P < 0.05$ was considered statistically significant.

RESULTS

Microarray analysis after GnRH treatment

Microarray analysis indicated that 31,873 genes of 55,681 genes examined were expressed in GT1-7 cells. Among the 31,873 genes, 257 genes and 165 genes were up-regulated and down-regulated, respectively, after GnRH treatment. Tables 2 and 3 list the 30 genes that were most strongly up-regulated and down-regulated, respectively, according to Z score. We excluded non-coding RNA genes from Tables 2 and 3. We performed an enrichment analysis of the 422 genes using DAVID software (6). Pathway analysis indicated that 11 genes among the 257 up-regulated genes were related to the MAPK signaling pathway. Among 11 genes, DUSP1, DUSP2, DUSP5, and DUSP6 were up-regulated 3.4-, 1.8-, 6.7-, and 3.8-fold, respectively, after GnRH treatment (underlined in Table 2).

Table 3 30 genes that were down-regulated by GnRH

Number	GeneSymbol	Gene Title	Z score	Ratio
1	Tcl1b3	T cell leukemia/lymphoma 1B	-12.29	0.00
2	Arhgef5	Rho guanine nucleotide exchange factor	-11.60	0.01
3	Mcf2l	Mcf.2 transforming sequence-like	-11.09	0.10
4	Ppp2r5e	Protein phosphatase 2, regulatory subunit B	-10.61	0.18
5	Vmn2r58	Vomerolateral 2, receptor 58	-10.44	0.01
6	Olfir312	Olfactory receptor 312	-8.96	0.02
7	Lmcd1	LIM and cysteine-rich domains 1	-8.85	0.02
8	Ttc14	Tetratricopeptide repeat domain 14	-8.70	0.25
9	Gabbr1	Gamma-aminobutyric acid (GABA) C receptor	-6.85	0.24
10	Mmrn1	Multimerin 1 (Mmrn1)	-6.13	0.02
11	Pten	Phosphatase and tensin homolog	-6.11	0.57
12	Pm20d1	Peptidase M20 domain containing 1	-5.92	0.02
13	Mrgprb4	MAS-related GPR, member B4	-5.87	0.07
14	Ntrk3	Neurotrophic tyrosine kinase, receptor, type 3	-5.77	0.30
15	Prl7c1	Prolactin family 7, subfamily c, member 1	-5.73	0.03
16	Pax6	Paired box gene 6	-5.34	0.33
17	Ccdc48	Coiled-coil domain containing 48	-5.00	0.04
18	Syt10	Synaptotagmin X	-4.87	0.64
19	Olfir601	Olfactory receptor 601	-4.82	0.05
20	Mirg	Maternally-expressed gene 9	-4.59	0.65
21	Olfir67	Olfactory receptor 67	-4.43	0.06
22	Krtap1-5	Keratin associated protein 1-5	-4.27	0.07
23	Wdr62	WD repeat domain 62	-4.20	0.15
24	Fgl2	Fibrinogen-like protein 2	-4.10	0.08
25	Ptn	Pleiotrophin	-3.99	0.16
26	Bambi	BMP and activin membrane-bound inhibitor	-3.94	0.09
27	Gabra6	Gamma-aminobutyric acid (GABA) A receptor	-3.79	0.10
28	C2cd4a	C2 calcium-dependent domain containing 4A	-3.73	0.10
29	Fam196b	Family with sequence similarity 196, member B	-3.66	0.47
30	Clec4b1	C-type lectin domain family 4, member b1	-3.62	0.11

Increase in the mRNA levels of DUSP5 and DUSP6 by GnRH treatment

We next examined whether or not the mRNA levels of DUSPs were increased by GnRH treatment using RT-PCR (Fig. 1). Although microarray analysis suggested that DUSP1 and DUSP2 genes were up-regulated after GnRH treatment, the mRNA levels of DUSP1 and DUSP2 were not increased clearly until 6 h. In contrast, the mRNA levels of DUSP5 and DUSP6 were increased from 30 min after GnRH treatment. The DUSP5 mRNA level peaked at 1–3 h, whereas the increase in DUSP6 mRNA level was transient, peaking at 30 min and declining by 1 h. The mRNA levels of DUSP5 and DUSP6 were increased approximately 3.6-fold at 1 h and 8.6-fold at 30 min, respectively. The mRNA level of GAPDH was not changed at any of the time points examined.

Increase in the protein levels of DUSP5 and DUSP6 by GnRH treatment

We intended to confirm that GnRH treatment in-

Fig. 1 Effects of GnRH on the levels of mRNAs of DUSP family genes. GT1-7 cells were treated with 50 nM GnRH for the periods indicated. After total RNA from GT1-7 cells was reverse transcribed, PCR was carried out using the primers against DUSP1, DUSP2, DUSP5, DUSP6, and GAPDH, as described in the MATERIALS AND METHODS. The positions of DUSP1, DUSP2, DUSP5, DUSP6, and GAPDH are indicated.

Fig. 2 Increase in DUSP5 and DUSP6 and activation of ERK by GnRH, and the effects of U0126 on GnRH actions. **A)** GT1-7 cells were treated with 50 nM GnRH for the periods indicated. Cell extracts (47 μ g) were subjected to SDS-PAGE in 10% acrylamide, and immunoblotting analysis was performed using an anti-DUSP6 antibody (1 : 700), an anti-active ERK antibody (1 : 850), or an anti-ERK antibody (1 : 1,500). The positions of DUSP6, ERK1 (P-ERK1, ERK1), and ERK2 (P-ERK2, ERK2) are indicated. **B)** GT1-7 cells were treated with 50 nM GnRH for the periods indicated. Cell extracts (47 μ g) were subjected to immunoblotting analysis as described above using an anti-DUSP5 antibody (1 : 200). HL-60 cell lysate was used as a positive control. The position of DUSP5 is indicated. **C)** GT1-7 cells were pretreated with or without 10 μ M U0126 for 30 min and treated with or without 50 nM GnRH for 30 min. After total RNA from GT1-7 cells was reverse transcribed, PCR of DUSP5, DUSP6, and GAPDH was performed as described in the MATERIALS AND METHODS. The positions of DUSP5, DUSP6, and GAPDH are indicated.

creased DUSP5 and DUSP6 at the protein levels. When we used an anti-DUSP6 antibody, two protein bands (42 k and 44 k) were observed (Fig. 2A). It was reported that the anti-DUSP6 antibody reacted with 42 k and 44 k proteins in primary murine embryonic fibroblasts by the manufacturer. Because the predicted molecular weight of mouse DUSP6 from the cloned sequence is 42 k, the 44 k protein may be a posttranslationally modified form of DUSP6 (2). We found that both the 42 k protein and the 44 k protein were increased from 1 h, and the increase in the DUSP6 proteins was sustained until 9 h. We obtained commercially two types of antibodies against DUSP5 to perform immunoblotting analysis. How-

ever, the antibodies did not react with DUSP5 in our assay conditions (data not shown). Because the antibodies were raised against human DUSP5 at the N-terminus and the C-terminus, respectively, these antibodies may not react with mouse DUSP5.

We next examined the time course of ERK activation after GnRH treatment (Fig. 2A). We commercially obtained an anti-ERK antibody and an anti-active ERK antibody that were polyclonal rabbit antibodies. An anti-ERK antibody was developed in rabbit using a synthetic peptide (amino acids 317–339) of human ERK1. An anti-active ERK antibody was affinity-purified using a dually phosphorylated synthetic peptide. The phosphorylated amino acid residues

correspond to threonine 183 and tyrosine 185 of ERK2. The amino acid sequences of both synthetic peptides are highly conserved in ERK1 and ERK2 and are identical in human, rat, and mouse ERK1 and ERK2. Immunoblotting analysis with an anti-ERK antibody indicated that ERK1 and ERK2 were expressed at similar levels in GT1-7 cells (Fig. 2A). ERK2 was more strongly activated than ERK1 by GnRH treatment. When we measured the activation level of ERK, we combined the activation levels of ERK1 and ERK2. ERK activation peaked at 5 min, and declined from 30 min. ERK was activated about 27-fold after 5-min treatment with GnRH. The activation level of ERK at 1 h was approximately 20% of that of ERK at 5 min. The protein amounts of ERK1 and ERK2 were not changed at any of the time points examined.

Because the commercially available antibodies against human DUSP5 did not react with DUSP5 in GT1-7 cells, we decided to produce an anti-DUSP5 antibody. We confirmed that the purified antibody detected a DUSP5 peptide until 10,000-fold dilution by ELISA (data not shown). Fig. 2B shows that several protein bands were detected in both GT1-7 cell lysate and HL-60 cell lysate. In addition, the anti-DUSP5 antibody strongly reacted with DUSP5 in HL-60 cell lysate at the predicted molecular weight, 44 k. The anti-DUSP5 antibody reacted with DUSP5 in GT1-7 cells, and DUSP5 protein was increased approximately 1.4-fold at 1 h after GnRH treatment of GT1-7 cells. The increase in DUSP5 protein peaked at 6 h and declined at 9 h.

Involvement of the ERK pathway in GnRH-induced increases of DUSP5 and DUSP6

Next, we asked whether or not the increases in DUSP5 and DUSP6 mRNAs were induced through the activation of the ERK pathway. GnRH treatment increased the mRNA levels of DUSP5 and DUSP6 approximately 3.3- and 4.0-fold, respectively (Fig. 2C). U0126, an inhibitor of ERK activation, inhibited the GnRH-induced increase in the mRNA levels of DUSP5 and DUSP6 almost completely. These results indicated that the ERK pathway was necessary for the GnRH-induced increases in DUSP5 and DUSP6 mRNAs. When the mRNA level of GAPDH was examined, no significant changes were observed for any treatment.

Involvement of DUSP6 in the deactivation of ERK after GnRH treatment

We considered the possibility that DUSP5 and DUSP6 might dephosphorylate ERK to form a neg-

ative feedback loop. Because DUSP6 was induced more robustly than DUSP5 by RT-PCR and immunoblotting analysis, we first knocked down DUSP6 and then treated the cells with GnRH (Fig. 3A). GnRH treatment activated ERK approximately 21.2-fold and 4.0-fold at 5 min and 1 h, respectively. Knockdown of DUSP6 did not significantly augment GnRH-induced ERK activation at 5 min, and ERK was activated approximately 22.4-fold in the presence of DUSP6 siRNA. In contrast, ERK was activated approximately 6.6-fold at 1 h after GnRH treatment in the presence of DUSP6 siRNA, suggesting that knockdown of DUSP6 augmented ERK activation approximately 1.7-fold compared with the presence of nonspecific siRNA. GnRH treatment for 1 h increased DUSP6 protein approximately 3.6-fold and 1.5-fold in the absence and presence of DUSP6 siRNA, respectively (Fig. 3A). When an anti-GAPDH antibody was used for immunoblotting analysis, no significant changes in immunoreactivity were observed for any treatment. Next, we knocked down both DUSP5 and DUSP6 and examined the GnRH-induced ERK activation (Fig. 3B). In this experiment, GnRH treatment activated ERK approximately 14.4-fold and 5.2-fold at 5 min and 1 h, respectively. After the knockdown of both DUSP5 and DUSP6, ERK was activated 14.9-fold and 6.2-fold at 5 min and 1 h, respectively. These results suggested that DUSP5 knockdown had no additive effects to DUSP6 knockdown on GnRH-induced ERK activation.

DISCUSSION

It has been reported that differences in the duration of ERK activation regulate signaling specificity (for review, see Ref. 7). In the case of a pheochromocytoma cell line, PC12 cells, epidermal growth factor (EGF) induces transient activation of the ERK pathway and stimulates the proliferation of PC12 cells (for review, see Ref. 1). In contrast, nerve growth factor induces sustained activation of the ERK pathway, and stimulates the differentiation of PC12 cells. A computer simulation of EGF-induced activation of ERK pathway indicated that feedback inhibition of ERK is the most important factor in determining the duration of ERK activation (1). It is well-known that GnRH treatment of GT1-7 cells induces transient activation of ERK (Fig. 2A). However, feedback inhibition of the ERK pathway in these cells has not been reported thus far.

In the present study, microarray analysis suggested that 4 types of DUSPs were up-regulated by GnRH treatment. In addition, we confirmed that GnRH

Fig. 3 Effects of DUSP5 and DUSP6 siRNAs on GnRH-induced ERK activation and DUSP6 expression. **A**) GT1-7 cells were transfected with 20 nM control siRNA or DUSP6 siRNA. After a 48-h incubation, the cells were treated with or without 50 nM GnRH for 5 min or 1 h. Cell extracts (15 µg) were subjected to SDS-PAGE in 10% acrylamide, and immunoblotting analysis was performed using an anti-active ERK antibody (1 : 850), an anti-DUSP6 antibody (1 : 600), or an anti-GAPDH antibody (1 : 1,000). **B**) GT1-7 cells were transfected with 20 nM control siRNA or DUSPs 5 and 6 siRNAs. After a 48-h incubation, the cells were treated with or without 50 nM GnRH for 5 min or 1 h. Cell extracts (15 µg) were subjected to SDS-PAGE and immunoblotting analysis as described above using an anti-active ERK antibody (1 : 850) or an anti-ERK antibody (1 : 1,500). The positions of ERK1 (P-ERK1, ERK1), ERK2 (P-ERK2, ERK2), DUSP6, and GAPDH are indicated.

treatment increased DUSP5 and DUSP6 at both the mRNA and protein levels. To the best of our knowledge, this is the first report of the up-regulation of DUSP5 and DUSP6 in GnRH neurons.

Because ERK activity declined within 1 h, and DUSP5 and DUSP6 proteins were increased within 1 h, we examined the possibility that DUSP5 and DUSP6 might play important roles in the feedback inhibition of ERK. We found that up-regulation of DUSP5 and DUSP6 by GnRH was dependent on the ERK pathway (Fig. 2C). In addition, knockdown of DUSP6 augmented the GnRH-induced activation of ERK at 1 h but not at 5 min (Fig. 3). These results suggested that a negative feedback mechanism of the ERK pathway by DUSP6 exists in GnRH neurons to regulate the duration of ERK activation

(Fig. 4). This feedback mechanism may be one of the reasons why GnRH-induced ERK activation is transient in GT1-7 cells.

We found that the increase in the mRNA level of DUSP6 was transient and declined from 1 h. In contrast, the increase in DUSP6 protein was sustained until 9 h. These results may suggest that ERK activation was inhibited for long period after gene expression of DUSP6. In the present study, we did not observe the knockdown effects of DUSP5 on ERK activation. It has been reported that DUSP6 is localized in the cytosol, whereas DUSP5 is localized in the nucleus (Fig. 4). DUSP6 may bind to the activated ERK through a MKB domain to inhibit the translocation of ERK from the cytosol to the nucleus. If this is the case, DUSP5 in the nucleus may

Fig. 4 Schematic representation of a negative feedback mechanism of the ERK pathway through DUSP6 in GnRH neurons.

not be able to dephosphorylate ERK.

It has been reported that GnRH treatment of cultured gonadotrophs up-regulated DUSP1 and DUSP2 (14, 17). DUSP1 inhibited ERK-mediated expression of LH β and FSH β genes (14). However, up-regulation of DUSP1 and DUSP2 after GnRH treatment of GT1-7 cells was not observed by RT-PCR in our assay conditions (Fig. 1). It is not clear at present whether or not these differences in the up-regulation of DUSP family by GnRH are due to the differences between gonadotrophs and GnRH neurons. Investigation of up-regulation of DUSP5 and DUSP6 after GnRH treatment of gonadotrophs is worth examining in a future study.

DUSP6 is reportedly the only DUSP that dephosphorylates ERK5 (7). Therefore, DUSP6 may dephosphorylate ERK5 in addition to ERK1 and ERK2 in GT1-7 cells. We confirmed that GT1-7 cells expressed ERK5, whereas ERK5 was not activated after GnRH treatment (Higa-Nakamine *et al.*, unpublished observation). Therefore, it is unlikely that DUSP6 dephosphorylates ERK5 after GnRH treatment. It is challenging to investigate the extracellular signals that activate ERK5 in GT1-7 cells and its dephosphorylation by DUSP6. In addition, elucidation of the molecular mechanisms for the up-regulation by

GnRH of DUSP6 in GT1-7 cells is crucial for future studies.

In conclusion, the present study contributes to the understanding of the regulation of the functions of GnRH neurons by GnRH. It is well-known that the precise regulation of the synthesis and secretion of LH and FSH in pituitary gonadotrophs is crucial for the female reproductive functions (for review, see Ref. 13). Because GnRH is the most important factor in the regulation of the synthesis and secretion of LH and FSH, the present study will contribute also to the understanding of the regulation of female reproductive cycles.

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CONFLICTS OF INTEREST

The authors declare that no significant conflicts of interest exist with any companies/organizations whose products or services may be discussed in this article.

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