MINI REVIEW

Characterization of Two FSH Inducible Genes, a Nuclear Transcriptional Co-activator p120 and a Novel Serine Protease-like Protein, in the Rat Ovarian Granulosa Cells

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Abstract

Ovarian follicular development is primarily dependent on pituitary gonadotropins. We report here the identification of two genes, a nuclear transcription co-activator p120, and a novel serine protease-like protein, as FSH inducible genes in the rat granulosa cells. Characterization of the genes revealed that rat p120 may work together with PR in the granulosa cells of large antral follicles to promote the ovulation events, and that the novel serine protease-like protein may be involved in the proteolytic process during the follicular development and ovulation process.

Introduction

Follicular growth is primarily controlled by pituitary gonadotropins, LH and FSH. Especially FSH stimulates proliferation and differentiation of granulosa cells of small follicles in the ovary, and promotes their development to pre-ovulatory follicles [1]. Recently we identified many gonadotropin inducible genes expressed in the ovary by subtraction cloning and DNA microarray analyses, and found that a transcriptional co-activator p120 and a novel serine protease-like protein were strongly induced in the rat cultured granulosa cells by

FSH. In this report, characterization of the co-activator p120 and the novel serine protease-like protein in the ovary will be described.

Subtraction cloning and DNA microarray

The subtraction cloning and DNA microarray technology are quite powerful methods for the comprehensive analysis of gene expression. In order to identify FSH-inducible genes in the rat granulosa cells, subtraction cloning and DNA microarray analyses were performed. Cultured granulosa cells $(5.0 \times 10^6$ viable cells) were treated with or without 30 ng/ml FSH for 15 hours. Messenger RNAs from FSH treated cells as well as control cells were converted to double stranded cDNAs, and subtraction cloning was performed as previously reported [2]. Resulting cDNAs of size-range between 0.5-2.0 kbp long were subcloned to construct a subtraction plasmid cDNA library. About 400 clones in the plasmid library were randomly picked up and their nucleotide sequences were partially determined. For DNA microarray analysis, mRNAs from the cells treated with or without FSH were labeled with fluorescent dyes, Cye3- or Cye5, respectively. The labeled cDNA pools were hybridized to microarray glass slides containing 3200 cDNA elements. As listed in Table 1, hundreds of candidate genes were picked-up and 10 genes were confirmed as FSH inducible genes by the semi-quantitative RT-PCR analysis. Among these FSH-inducible genes, we describe here the expression and localization of the transcriptional co-activator p120, as well as those of the novel serine protease-like

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Table 1. Induced genes in the rat granulosa cells stimulated with FSH for 15 hours

Accession number	Definition
AB180485	Human thyroid hormone receptor coactivating protein like protein (rat p120)
AB180912	Unknown serine protease like protein
NM_017290	Rat ATPase, Ca^{2+} transporting, cardiac muscle, slow twitch 2 (Atp2a2)
NM_022265	Rat programmed cell death 4 (Pdcd4)
M64393	Rat 3-α-hydroxysteroid dehydrogenase (3-α-HSD)
NM_019683	Mouse globin inducing factor, fetal (Gbif-pending)
NM_011462	Mouse spindlin (Spin)
U23776	Rat Eker rat-associated intracisternal-A particle element
NM_012600	Rat Malic enzyme 1, soluble (Me1)
AY009092	Rat retrovirus SC1

protein in the ovary during the follicular development and the ovulation processes.

Characterization of rat p120, an FSH inducible transcriptional co-activator

In this study we identify rat homologue of p120, a nuclear transcription co-activator, as one of the FSH inducible genes in the rat granulosa cells. We then cloned a full-length cDNA encoding rat p120 and the nucleotide sequence was determined. Fig. 1 shows the deduced amino acid sequence of rat p120, which is compared with that of human p120. It contains proline-rich, acidic, and bromo-domains, respectively, and there are two LXXLL-motifs between proline-rich and acidic domains. The sequences are well conserved between rat and human (94% identity at amino acid level), except that rat p120 has 45 amino acid residues extended at N-terminus. The transcriptional co-activator p120 was originally isolated as a novel nuclear co-activator for thyroid hormone receptor [3]. Further studies revealed that p120 also interacts with androgen receptor or 9-cis-retinoic acid receptor (RXR)

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Rat      1   MLLQRVNLCS  VSGTPEVWPL  WGLSTVISFR  VVPGSNSGCR  AFRTRVSVSR  AIKPFAEPGR   60
Human    1                                      MRS  GDQNW*****  *********   23

Rat     61   PPDWFSQKHC  ASQYSELLET  TETPKRKRGE  KGEVVETVED  VIVRKLTAER  VEELKKVIKE  120
Human   24   **********  **********  **********  **********  **********  **********   83

Rat    121   TQERYRRLKR  DAELIQAGHM  DSRLDELCSD  IAMKKKLEEE  EAEVKRKATD  AAYQARQAVK  180
Human   84   **********  **********  ********N*  **T*******  **********  **********  143

Rat    181   TPPRRLPTVM  VRSPIDSASP  GGDYPLGDLT  PTTMEEATSG  VTPGTLPSTP  VTSFPGIPDT  240
Human  144   **********  **********  **********  **********  **********  **********  203

Rat    241   LPPGSAPLEA  PMTPVTDDSP  QKKMLGQKAT  PPPSPLLSEL  LKKGSLLPTS  PRLVNESEMP  300
Human  204   **********  **********  **********  **********  **********  ********A  263

Rat    301   VPSGHLSSTG  VLLEVGGVLP  MIHGGDIQPA  TSAVAASPAA  SGAPTLSRLL  EAGPTQFSTP  360
Human  264   *A****N***  **********  *****E**QT  PNT*******  **********  *******T**  323

Rat    361   LPSFTTVASE  PPVKLVPPPV  ESVSQATIVM  MPALPAPSSA  PAVSTPESVA  PVSQPEPCVP  420
Human  324   *A********  **********  ****H*****  **********  *****T****  *****DN***  383

Rat    421   LEAVGDPQTV  TVSMDSSEIS  MIINSIKEEC  FRSGVAEAPG  GSKAPSIDGK  EDLDLAEKMD  480
Human  384   M******H**  **********  **********  ********V  **********  *E********  443

Rat    481   IAVSYTGEEL  DFETVGDIIA  IIEDKVDDHP  EVLDVAAVEA  ALSFCEENDD  PQSLPGPWEH  540
Human  444   **********  **********  **********  **********  **********  **********  503

Rat    541   PIQQERDKPV  PLPAPEMTVK  QERLDFEESE  TKGIHELVDI  RDSGVEIKVE  PTEPEPGMSG  600
Human  504   **********  **********  ********T*  N*********  *EPSA*****  *A****VI**  563

Rat    601   AEIVAGVGPV  PSLEPPELRS  QDLDEEPRSS  AAGEMGEADV  SSGKGEERPL  PAVKTEASPE  660
Human  564   *******V*A  T*M*******  ******LG*T  ****IV***  AI***D*T**  TN********  623

Rat    661   SMLSPSHGSN  PIEDPLEAET  QHKFEMSDSL  KEESGTIFGS  QIKDAPGEEE  EEDGVSEAAS  720
Human  624   **********  **********  **********  **********  ********D*  **********  683

Rat    721   LEEPKEEDQG  EGYLSEMDNE  PPVSESDDGF  SIHNATLQSH  TLGDSIPSSP  ASSQFSVCSE  780
Human  684   **********  **********  **********  **********  **A*******  **********  743

Rat    781   DQEAIQAQKI  WKKAIMLVWR  AAANHRYANV  FLQPVTDDIA  PGYHSIVQRP  MDLSTIKKNI  840
Human  744   **********  **********  **********  **********  **********  **********  803

Rat    841   ENGLIRSTAE  FQRDIMLMFQ  NAVMYNSSDH  DVYHMAVEMQ  RDVLEQIQQF  LATQLIMQTS  900
Human  804   **********  **********  **********  **********  **********  **********  863

Rat    901   ESGISAKSLR  GRDSTRKQDA  SEKDSVPMGS  PAFLLSLFDG  GTRGRRCAIE  ADMKMKK*   957
Human  864   **********  **********  **********  **********  **********  *******   920
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Fig. 1. Deduced amino acid sequence of rat p120
The proline-rich domain between 223 and 288 amino acid residues from the N-terminus is underlined, and acidic amino acid-rich domain between 602 and 731 is shown by bold type. The bromodomain between 809 and 894 is boxed. The putative NR box (LXXLL motif), which is necessary for the interaction with nuclear hormone receptors, is boxed by colored background. Since there are high homology between rat and human p120 sequence (94% identity at amino acid level), rat p120 is expected to have similar functions.

functioning as a co-activator of these nuclear receptors [4]. The receptors belong to the nuclear receptor super-family proteins that activate the transcription of their target genes when the steroid hormones or ligands bind to them. The nuclear hormone receptors generally bind to the respective hormone responsive elements (HREs) in the upstream regions of the target genes, and activate the transcription. Deficiencies of estrogen receptors or progesterone receptors (PR) result in various defects of reproductive functions, such as abnormal follicular development, anovulation, and infertility [5-7]. Transcriptional co-activators are required to form active protein complexes with the hormone nuclear receptors and the basic transcription machinery proteins for the target gene transcription. Co-activators p160/SRC and CBP/p300 are known to interact with the nuclear hormone receptors at the C-terminal AF-2 region through the LXXLL-motif (NR box) [8-10]. Knock-out mice of steroid receptor co-activator 3 (SRC-3), a member of p160 family, exhibit infertility [11], and those of nuclear receptor interacting protein 1 (Nrip1/RIP140) are also infertile due to the blockage of the oocytes release from the Graafian follicles by the ovulatory stimuli [12]. These reports indicate that co-activators are also important for the follicular development and ovulation. Therefore we examined expression and localization of p120 gene in the ovary during the follicular development using immature rats primed with gonadotropins. As shown in Fig. 2A, in situ hybridization revealed that the induction pattern of p120 was very similar to that of PR in the granulosa cells of large pre-ovulatory follicles. It is well established that PR participates in the ovulation process occurring in the pre-ovulatory large antral follicles. Ovulation is triggered by LH surge from the pituitary. About 4 hours after the LH surge, expression of PR is rapidly induced in the granulosa cells of pre-ovulatory large antral follicles destined to ovulate [13, 14]. Progesterone binds to the induced receptor to activate gene expression of specific proteases, ADAMTS-1 or Cathepsin L, which are thought to work on follicle rupture by degrading the ovulating follicle walls [15]. In addition, we examined co-activator functions of rat p120 by co-transfection experiments to show that p120 may promote the action of PR during the ovulation. As
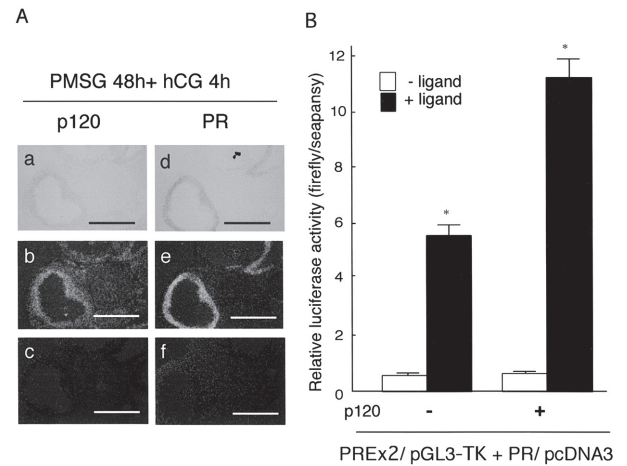

Fig. 2. Localization and transcriptional co-activator function of rat p120
(A) Sections of ovaries from immature rats primed with hCG for 4 hours followed PMSG 48 hours were hybridized with p120 (a, b; antisense probe, c; sense probe) or PR (d, e; antisense probe, f; sense probe). Expression of these genes was completely co-localized in the granulosa cells of large antral follicles, suggesting that p120 works together with PR in the ovary.
(B) Transcriptional co-activator activity of rat p120 was examined by co-transfection experiments. CV-1 cells were transfected with a luciferase reporter plasmid and the human PR expression vector with or without 10^{-7}M progesterone, in the presence or absence of p120. The luciferase activity of PR was upregulated about two fold in the presence of p120.

shown in Fig. 2B, p120 enhanced the transcription activity of PR in the presence of the ligand progesterone. These observations suggest that p120 may work together with PR in the granulosa cells of ovulatory follicles to promote the ovulation in the ovary.

A novel serine protease-like protein in the ovary

We also examined expression and localization of the novel FSH-inducible serine protease-like protein in the ovary during the follicular development, in order to check possible involvement of the gene in some ovarian proteolytic processes. The extracellular matrix (ECM) influences basic cellular processes such as proliferation, differentiation, migration and adhesion, and is fundamental to normal development. A tightly regulated proteolytic activity controls the remodeling and degradation of matrix components in these processes. In the ovary, many physiological and pathological processes including follicular development, ovulation and

angiogenesis require a regulated turnover of ECM components. Although a lot of proteolytic enzymes including members of the plasminogen activator (PA) or matrix metalloproteinase (MMP) have been recognized to be involved in several ovarian proteolytic events, a definitive functional role for individual proteases has yet to be demonstrated [16]. Indeed, there exist many deficient mouse strains that lack individual matrix degrading proteases, but these mice are fertile and don't have major defects of ovulation and female reproduction, as shown in knockout mice of PA [17].

In this context, we cloned a full-length cDNA encoding a novel FSH inducible ovarian serine protease-like protein and the nucleotide sequence was determined. Fig. 3 shows the nucleotide sequence of the serine protease-like protein along with deduced amino acid sequence. The gene encodes a protein of 406 amino acid

residues with a putative signal sequence, and putative active site Asp, His, and Ser residues that are conserved in serine protease family such as PA protein. As shown in Fig. 4, RT-PCR revealed that the serine protease was expressed in the brain (lane 1), adrenal (lane 4), intestine (lane 9), testis (lane 6), and ovary (lane 11). PMSG treatment increased the gene expression in the ovary after 48 hours (lane 12).

Northern blot analysis revealed that the serine protease mRNA level was rapidly induced by the FSH treatment within 3 hours, and the levels were kept high throughout the treatment (Fig. 5A). As shown in Fig. 5B, the treatment of immature rats with PMSG in
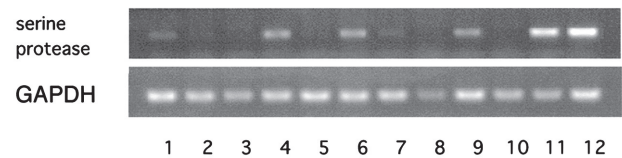

Fig. 4. Expression of the serine protease-like protein gene in various tissues
RT-PCR was performed using various tissues. The samples are brain (lane 1), pituitary (lane 2), liver (lane 3), adrenal (lane 4), uterus (lane 5), testis (lane 6), kidney (lane 7), spleen (lane 8), intestine (lane 9), stomach (lane 10), untreated ovary (lane 11), ovary treated with PMSG for 48 hours (lane 12).

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ATGTTACTTTGGCTGATAATTTTCGTCTCTGGATGGACCCTGTCTTTGGGATCTGAAACG     60
 M  L  L  W  L  I  I  F  V  S  G  W  T  L  S  L  G  S  E  T      20

GAACCAGATTTTACGTGGCACCTGAGCCGAATACCCCAGGTTGTGAGTGAGAAGACGATC    120
 E  P  D  F  T  W  H  L  S  R  I  P  Q  V  V  S  E  K  T  I      40

CACCTTGCCAGCCCCACGTTCCAGGCAGACGCTGCGGCGGTCAAGGCCACGGTGTGTGGC    180
 H  L  A  S  P  T  F  Q  A  D  A  A  A  V  K  A  T  V  C  G      60

ATCGAATGTCAGGAAGAGCTCCCGGCTCCCAGCCTCTCCCAGCTGGAAGATTTCCTTTCC    240
 I  E  C  Q  E  E  L  P  A  P  S  L  S  Q  L  E  D  F  L  S      80

TACGAGACGGTCTTCGAGAATGGCACTCGAACCTTAACCAGGGTCAAAGTTCAAGGTCTG    300
 Y  E  T  V  F  E  N  G  T  R  T  L  T  R  V  K  V  Q  G  L     100

GTCTTGGAACCCACTCAGAACAGCAGCATAAAAGGAGCACGCCCTAGGAGAAGAAGGCAG    360
 V  L  E  P  T  Q  N  S  S  I  K  G  A  R  P  R  R  R  R  Q     120

GTGTACGGTACCGACAGCAGGTTCAGCATCTTAGACAAAAGGTTCTTGACCAATTTCCCT    420
 V  Y  G  T  D  S  R  F  S  I  L  D  K  R  F  L  T  N  F  P     140

TTTAATACAGCGGTGAAGCTGTCCACCGGCTGCAGCGGCGCCCTCGTTTCCCCCAACCAC    480
 F  N  T  A  V  K  L  S  T  G  C  S  G  A  L  V  S  P  N  H     160

GTGCTCACGGCTGCCCACTGTGTCCACGACGGGAAGGACTATGTCAAAGGTAGTAAAAAG    540
 V  L  T  A  A  H  C  V  H  D  G  K  D  Y  V  K  G  S  K  K     180

CTGAGGGTGGGAGTGCTGAAGATGAGAAATAAAGGAGGCCGCAAGAAACGCAGAGGTTCC    600
 L  R  V  G  V  L  K  M  R  N  K  G  G  R  K  K  R  R  G  S     200

AGGAGGAGCCGGCGAGAAGCAGAGAGTGGTGGCCAAAGTCCGGAGCATCCTCAGGAGAGC    660
 R  R  S  R  R  E  A  E  S  G  G  Q  S  P  E  H  P  Q  E  S     220

ACCACCCAAAGACCAGGGAAAAAATCCAGGCGGGGTCCTAGAGTCGCCCAAGGAAGGCCT    720
 T  T  Q  R  P  G  K  K  S  R  R  G  P  R  V  A  Q  G  R  P     240

TCCTTCCAATGGACCCGCGTCAAGAGCACCCACATTCCCAAAGGCTGGGCAAGGGGAGAG    780
 S  F  Q  W  T  R  V  K  S  T  H  I  P  K  G  W  A  R  G  E     260

AATGGGGACCCGGCCTTGGACTTCGACTATGCACTCCTGGAGCTGAAGCGCGCACAAAAG    840
 N  G  D  P  A  L  D  F  D  Y  A  L  L  E  L  K  R  A  Q  K     280

CAGCAGTACATGGAGCTGGGAGTCAGCCCCACCATCTCCAAGCTGCCGGGAGGTCGGATC    900
 Q  Q  Y  M  E  L  G  V  S  P  T  I  S  K  L  P  G  G  R  I     300

CACTTCTCTGGATTTGATAACGACAGGGATGATCAGTTGGTGTATCGGTTTTGCAGCGTT    960
 H  F  S  G  F  D  N  D  R  D  D  Q  L  V  Y  R  F  C  S  V     320

TCCGAGGAATCCAATGACCTCCTGTATCAGTACTGCGACGCTGAGGCAGGCTCCACCGGC   1020
 S  E  E  S  N  D  L  L  Y  Q  Y  C  D  A  E  A  G  S  T  G     340

TCTGGGATCTACCTGAGGCTCAAAGAGCCAGGCCAAAAGAATTGGAAGCGCAAGATCATC   1080
 S  G  I  Y  L  R  L  K  E  P  G  Q  K  N  W  K  R  K  I  I     360

GCGGTCTACTCGGGCCACCAGTGGGTGGATGTGCACGGAGTTCAGAAGGACTATAACGTG   1140
 A  V  Y  S  G  H  Q  W  V  D  V  H  G  V  Q  K  D  Y  N  V     380

GCTGTGCGCTATCACTCCGCTCAAGTACGCCCAGATTTGCCTCTGGATCCACGGAAACGCT   1200
 A  V  R  I  T  P  L  K  Y  A  Q  I  C  L  W  I  H  G  N  A     400

GCCAACTGTGCTTATGGCTGA                                           1221
 A  N  C  A  Y  G  *                                            406
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Fig. 3. Nucleotide and deduced amino acid sequence of the serine protease-like protein
The amino acids are numbered starting from the initiation codon. The deduced signal sequence between 1 and 17 residues from the N-terminus is underlined, and conserved residues between 162 and 167 involving the putative active site histidine residue is boxed.

A. granulosa cell

serine protease

GAPDH

FSH
0 1.5 3 6 12 24 h

B. ovary

serine protease

36B4

PMSG
0 3 6 12 24 48 h

C. ovary

serine protease
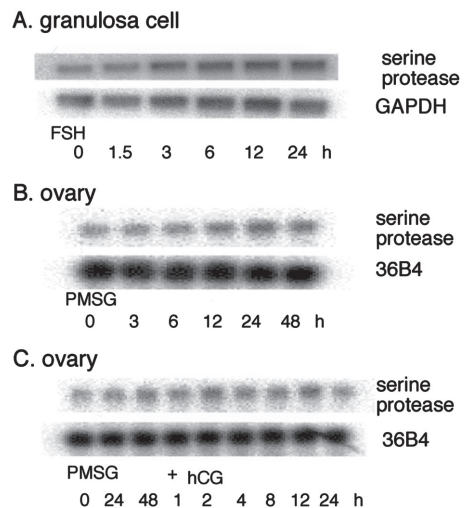
36B4

PMSG   + hCG
0 24 48 1 2 4 8 12 24 h

Fig. 5. Induction of the serine protease-like protein gene expression in the ovary
Northern blot analysis of the serine protease-like protein mRNA in cultured granulosa cells treated with FSH (A), in immature rat ovaries primed with PMSG (B), and in immature rat ovaries primed with PMSG followed by hCG (C).

vivo also induced the protease gene expression in the ovary within 6 hours after the treatment, with a transient peak at 24 hours after the treatment. Further hCG treatment also increased the gene expression with a peak at 2 hours as shown in Fig. 5C.
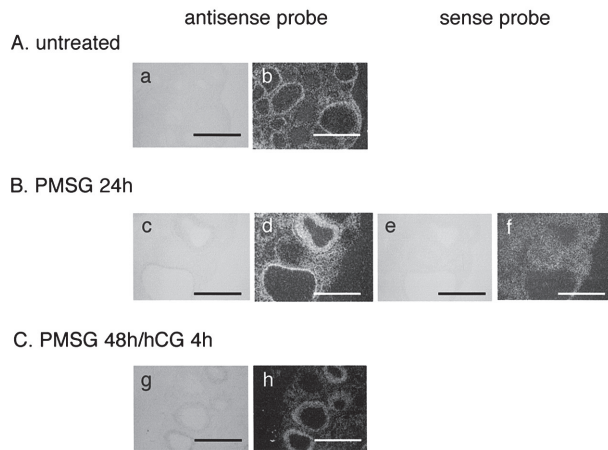

Fig.6. In situ hybridization of the serine protease-like protein
Ovaries from 21-d-old immature rats were used. (A) Control ovaries were hybridized with the antisense probe of the serine protease-like protein (a, b), (B) those treated with PMSG for 24 hours were hybridized with the antisense probe (c, d), or hybridized with sense probes (e, f), (C) those treated with hCG for 4 hours after the PMSG treatment were hybridized with the antisense probe (g, h). Original magnification was x100. Scale bars, 0.1 mm.
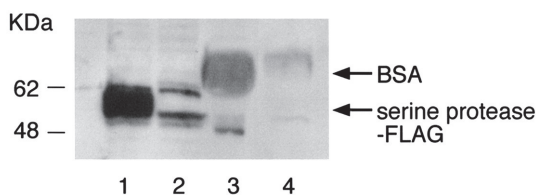

Fig.7. Recombinant expression of the serine protease-like protein
CHO cells were transfected with 10 μg of the serine protease-FLAG/pcDNA3 construct or with control pcDNA3 vector. After 24 hours, culture medium were collected and concentrated. Cell lysates were also prepared. Samples were resolved on a 10 % SDS-PAGE gel and immunoblotted with the M2 anti-FLAG epitope antiserum (Sigma). Cell lysate from the serine protease-like protein-FLAG/pcDNA3 transfected cells (lane 1), from the control vector transfected cells (lane 2), the concentrated culture medium from the serine protease-like protein-FLAG/pcDNA3 transfected cells (lane 3), and the concentrated culture medium from the control vector transfected cells (lane 4). A specific band corresponding the recombinant protein was detected at 55 KDa. This indicates that the novel serine protease-like protein was actually translated in the cells, and may function as an ovarian protease.

Localization of the serine protease in the ovary was also examined by in situ hybridization using adjacent frozen sections as shown in Fig. 6. Immature female rats were treated either with PMSG or PMSG/hCG. The expression of the gene was induced in the granulosa cells of large antral follicles 4 hours after the hCG treatment (Fig. 6C). Since the treatment described above was typical for ovulation induction, the expression patterns of this serine protease-like protein suggest possible involvement of this protein in the ovulatory events. Expression of the serine protease was confirmed in transiently transfected CHO cells by Western blotting for FLAG epitope that engineered at the C terminus (Fig. 7). The detected band was consistent with the predicted molecular mass of 55 KDa. Further studies will be required to show the substrate specificity of this protein in order to clarify its physiological role during the follicle development. It is well known that ADAMTS-1 is multifunctional MMP and induced by LH surge in the granulosa cells and cumulus cells, and has a critical downstream role in mediating the PR-regulated ovarian activity that culminates in the rupture of a follicle [15]. The novel serine protease-like protein described here may also be a member of crucial proteases involved in such processes.

Conclusion

In this report, we described the isolation and the characterization of two FSH inducible genes in the rat ovarian granulosa cells. We showed here that rat p120, a transcriptional co-activator, interacts with PR to enhance their activity, and co-localized with PR in the granulosa cells of large antral follicles. Our observation suggests that the co-activator p120 may participate in the ovulation processes co-working with PR. We also identified a novel serine protease-like protein in the rat granulosa cells. Although further studies should clearly be needed for its role in the ovary, the novel serine protease-like protein may be a new crucial member of proteases involved in the proteolytic processes in the ovary.

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