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A Proline Rich Acidic Protein PRAP Identified From Uterine Luminal Fluid of Estrous Mice is Able to Enhance the Estrogen Responsiveness of Ishikawa Cells

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ABSTRACT

Using mice as experimental animals, proteins in the uterine luminal fluid (ULF) from both adults and diethylstilbestrol dipropionate (DES)-treated immature animals were resolved by 2D gel electrophoresis. Two of the protein spots, (a) and (b) around the positions of 18–20 kDa, in the adult ULF were not found in the DES-treated ULF. Automated Edman degradation established the same N-terminal sequences of AHQVPVKTKGKHVFP for the two protein spots. Two trypsin digests of spot (a) were analyzed using CID MS/MS to establish the peptide sequences DNQLGPLLPEPK and RPDAMTWVETEDILSHLR. These partial sequences were confirmed in the cDNA-deduced mouse proline rich acidic protein (PRAP). Using human Ishikawa cell line as a surrogate endometrial model, we demonstrated rapid entrance of exogenous PRAP into the cells and its ability to enhance alkaline phosphatase activity of the E_2-stimulated cells. Further, the transcripts of five estrogen-responsive genes, including *ALPP* (Placental alkaline phosphtase), *ALPPL* (placental alkaline phosphatase-like 2), *TGF* (transforming growth factor), *PR* (progesterone receptor), and *Wnt7a*, were measured after the cell incubation in modified Eagle medium containing 0.1 nM E_2, or 0–25 μM PRAP, or both together at 37°C for 48 h. As compared with the control, E_2 alone increased the transcripts of *ALPP*, *ALPPL*, *TGF-α*, and *PR,* and reduced the transcript of *Wnt7a*, whereas PRAP alone had a slight impact on their expression. E_2 together with PRAP greatly increased the E_2-stimulated transcriptions of *ALPP*, *ALPPL*, *TGF-α*, and *PR*, and markedly reduced the E_2-suppressed transcription of *Wnt*7a. J. Cell. Biochem. 112: 3122–3128, 2011. © 2011 Wiley Periodicals, Inc.

KEY WORDS: ENDOCYTOSIS; ENDOMETRIAL ESTROGEN RESPONSE; PROLINE RICH ACIDIC PROTEIN; PROTEOMICS; UTERINE LUMINAL FLUID

A proline-rich acidic protein, designated proline rich acidic protein (PRAP), is a conceptual product translated from a complementary DNA that was first identified in the mouse uterus during late pregnancy [Kasik and Rice, 1997]. Zhang et al. [2000] demonstrated the *PRAP* transcript in the epithelial cells of the mouse and rat gastrointestinal tracts, found the human homologue expressed in the epithelial cells of the human liver, kidney, gastrointestinal tract, and cervix, and correlated cancer cell growth with the down-regulation of *PRAP* expression through an epigenetic mechanism involving histone deacetylation and methylation [Zhang et al., 2003]. Yet, the functional role of this protein in mammalian reproduction has been not reported.

The mammalian uterine physiology is regulated by circulating ovarian steroids. Secretion of uterine luminal fluid (ULF) from estrous females, a marker of female sexual maturity, is estrogen dependent. In rodents, this occurs during the proestrous phase of estrous cycle [Albers and Neves, 1961]. The identification of ULF proteins and elucidation of their functions have become important in understanding their roles in mammalian reproduction. Unlike adult females, there is scant secretion of ULF from immature females. Since exposure of immature rodent to diethylstilbestrol dipropionate (DES), a well-known environmental chemical with potent estrogenic activity, increases an abundant secretion of ULF, they have been used as good experimental animals to study the ULF

Abbreviations: ALPP, Placental alkaline phosphatase; ALPPL, placental alkaline phosphatase-like 2; DES, diethylstilbestrol dipropionate; E2, estradiol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LCM, Laser Capture Microdissection; PRAP, proline rich acidic protein; PR, progesterone receptor; TGF, transforming growth factor; ULF, uterine luminal fluid.

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proteins. Using mice as an animal model, this work was conducted to examine the ULF proteins by proteomic analysis. We identified PRAP in ULF of estrous females, whereas it was absent in ULF of the DES-stimulated immature mice. Further, we demonstrated rapid entrance of PRAP in the milieu into Ishikawa cells, a surrogate endometrial model, and its ability to enhance the cellular estrogen response.

MATERIALS AND METHODS

CHEMICALS

Full Range Rainbow molecular-weight markers (Amersham Pharmacia Biotech, Uppsala, Sweden); BCA protein assay kit (Pierce, Rockford, IL); DES, estradiol (E_2), phenylmethylsulphonyl fluoride (PMSF) (Sigma Chemical Co., St Louis, MO); horseradish peroxidase-conjugated goat anti-rabbit IgG (Promega, Madison, WI); 4′6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA); enhanced chemiluminescent (ECL) substrate (NEN Life Science Products, Boston, MA); expression vector pET-21a (+), T7 promoter and terminator primers (Novagen, Madison, WI); Trypsin-EDTA solution, modified Eagle medium (MEM), fetal bovine serum (FBS), penicillin G, streptomycin, non-essential amino acids, and L-glutamine (GIBCO/BRL, Frederick, MD).

ANIMALS, DES TREATMENT, AND PREPARATION OF ULF

The care and use of outbred ICR mice (Charles River Laboratories, Wilmington, MA) followed the protocol RMiIBCCY2009080 approved by Academia Sinica, Taiwan. Immature (21-day-old) and sexually mature (6–8-week-old) females were used. The estrous cycle was staged by examining vaginal smears. DES was dissolved in corn oil used as a vehicle. Immature females received subcutaneous injection of DES ($0.1\,\mu g\,g^{-1}$ day^{-1}) for three consecutive days were sacrificed within 24 h after the last injection. ULF collected from the DES-treated immature females or estrous females during the proestrous phase of estrous cycle was mixed in a final concentration of 1.0 mM EDTA and 1.0 mM PMSF, centrifuged at 14,000*g* for 10 min, and stored at −80°C.

2-D ELECTROPHORESIS OF ULF PROTEINS AND DETERMINATION OF PEPTIDE SEQUENCE

The ULF sample was resolved using an IEF focusing system (Protean II XI Multi-cell, Bio-Rad, Richmond, CA) on a 7-cm IPG strip (Amersham Corp, Buckinghamshire, UK) at a linear gradient of pH 3.0–10.0. The voltage was slowly ramped up during IEF (10–16 h) to a maximum of 4.0 kV for a total 30 kVh. The proteins on the isoelectrofocused IPG were further resolved by SDS–PAGE on a 12% gel slab ($8 \times 6 \times 0.1$ cm). The gel was silver-stained according to a previous method [Hochstrasser et al., 1988].

The gels of spots (a) and (b) on Figure 1 were excised. The protein extract of each gel was subjected to SDS–PAGE on a 12% gel strip and transferred the protein bands to a Polyvinylidene Fluoride (PVDF) filter at 100 V for 1 h at 4°C [Towbin, 2009]. Automated Edman degradation of the protein sample on a membrane filter was performed by a Procise 492 protein sequencer (Applied Biosystem, Foster City, CA).

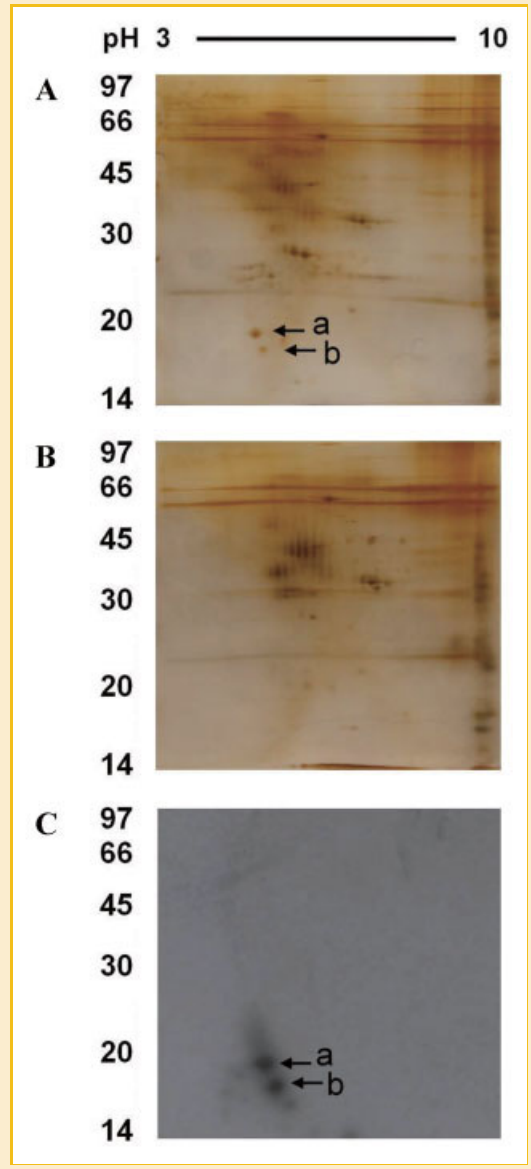

Fig. 1. Resolution of ULF proteins. ULF from adult (A) or DES-stimulated baby (B) was subjected to isoelectrofocusing, and then followed the SDS–PAGE on a 12% polyacrylamide gel slab. The silver-stained spots denoted by (a) and (b) on (A) were absent on (B). C: The proteins on (A) were immunoblotted with the antiserum against PRAP. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Following the method of Hellman et al. [1995], spot (a) in the gel was washed with 100 µl of 50% ACN/25 mM NH_4CO_3, dried, and incubated with trypsin (0.1 mg/ml) in 25 mM NH_4CO_3 at 37°C overnight. The trypsin digests were extracted with 50 µl of 50% ACN/5% TFA (v/v), dried, resuspended in 100 µl of 10% formic acid, desalted, and concentrated using Zip-Tip (Millipore, Bedford, MA). Sample plates were premixed 1: 1 with matrix solution (5 mg/ml CHCA in 50% ACN, 0.1% v/v TFA, and 2% w/v ammonium citrate), and samples were spotted onto the 96-well MALDI for MALDI MS and MS/MS analyses. Data-dependent MS/MS acquisition was performed on the Ultima MALDI-Q-TOF instrument (Waters,

Milford, MA). A survey scan m/z = 800–3000 was acquired and up to five of the highest-intensity precursors were selected for CID MS/MS using mass-dependent ±5 V rolling collision energy. The spectra were processed using the Micromass PLGS 2.2 software. Subsequently, the derived peak list was searched again with MSDB by the MASCOT search engine.

COMPUTATIONAL ANALYSIS

Six mammalian PRAP sequences were deduced from their GenBank cDNAs: Rattus norvegicus, NP_113857.1; Mus musculus, NP_033501.1; Homo sapiens, NP_660203.3; Canis familiaris, XP_850315.1; Bos Taurus, XP_590365.2; and Pan troglodytes, XP_001158828.1. The sequence alignments and phylogenetic tree were constructed by the neighbor-joining method using the Megalign program in the DNAStar software package [Xia and Xie, 2001].

CELL CULTURE

Ishikawa cells (European Collection of Cell Cultures, ECACC) were grown in a culture medium prepared by adding 5% (v/v) fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, non-essential amino acids, and L-glutamine to phenol red-depleted MEM. Cells were kept in plastic culture flasks under 5% CO_2 at 37°C, and harvested by a brief exposure to trypsin-EDTA solution at 37°C. The culture medium was added to stop the trypsin action before cell collection. Cells were re-suspended in the culture medium and seeded in 12-well plates at a density of 3.0×10^5 cells/well. Cells were exposed to E_2 and/or PRAP in a medium devoid of fetal bovine serum. After incubation at 37°C for 48 h, cells were harvested, suspended in 10% diethanolamine-0.5 mM $MgCl_2$ at pH 10.0, and homogenized at 4°C using ultrasonic disintegration.

RNA ISOLATION

Total RNA was extracted the homogenate of Ishikawa cells using an RNAqueous-4PCR kit (Ambion, Austin, TX). Each RNA preparation was treated with DNase and reverse-transcribed using ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA).

RT-PCR ANALYSIS OF ISHIKAWA CELL SAMPLES

Reverse-transcribed RNA sample of each Ishikawa cell sample (1.0 μg) was applied to quantitative RT-PCR (Q-PCR), which was performed in triplicate by a Lightcycler instrument with version 3.5 software using the LightCycler FastStart DNA Master SYBR Green I kit (Roche, Mannheim, Germany). The primer pair included: ^{199}CGGCAAATTCAACGGCACAGT^{219} (sense) and ^{755}TGGGGGTAGGAACACGGAAGG^{735} (antisense) in mouse *GAPDH* (GenBank XM_194302); ^{2251}CACCAGGGGGATTTTGACACAGC^{2273} (sense) and ^{2684}ACAGGCGCATAGATAAGGGGTTT-C^{2661} (antisense) in *ALPP* (GenBank NM_001632); ^{1907}TCAGGAAAAGAGGAGGCTCA^{1926} (sense) and ^{2269}TCTGAGTGGCTGTGACTTGG^{2250} (antisense) in *ALPPL* (GenBank NM_031313); ^{188}CTGCCCGCCCGCCCGTAAAAT^{208} (sense) and ^{716}CCACCTGGCCAAACTCCTCCTCTG^{693} (antisense) in *TGF-α* (GenBank NM_003236); ^{294}CACGAGTTTGATGCCAGAGA^{313} (sense) and ^{657}AGGGAGGAGAAAGTGGGTGT^{638} (antisense) in *PR* (GenBank NM_000926); ^{1055}CTTCCTGAAGATCAAGAAGCCACTGTC^{1081} (sense) and ^{1324}CTGCACGTGTTGCACTTGAC-ATAGCAG^{1298} (antisense) in *Wnt7a* (GenBank NM_004625); ^{443}GGGGAGCCAAAAGGGTCATCATCT^{466} (sense) and ^{899}GACGCCTGCTTCACCACCTTCTTG ^{876} (antisense) in human *GAPDH* (GenBank NM_002046).

ANTISERUM PREPARATION AND WESTERN BLOT ANALYSIS

Nucleotides 120–500 in mouse PRAP cDNA (GenBank NM _009475) was amplified from reverse-transcribed RNA sample of adult uteri by RT-PCR, using a primer pair including ^{120}GCACACCAGGTCCCTGTCAAG^{140} (sense) and ^{500}AGAATCCTCCATAGAGTGGGC^{480} (antisense) (Fig. 2A). The DNA fragment was ligated into pET21a expression vector via EcoR I and Xho I restriction sites. The constructed plasmids were used to transform the *E. coli* strain BL21 (DE3). The cells were induced by 0.4 mM IPTG at 37°C for 5 h. The his_6-tagged PRAP was purified by Ni-Sepharose 6 Fast Flow (GE Healthcare Bioscience, Princeton, NJ). The purity of the polypeptide was identified by 12% reducing SDS–PAGE.

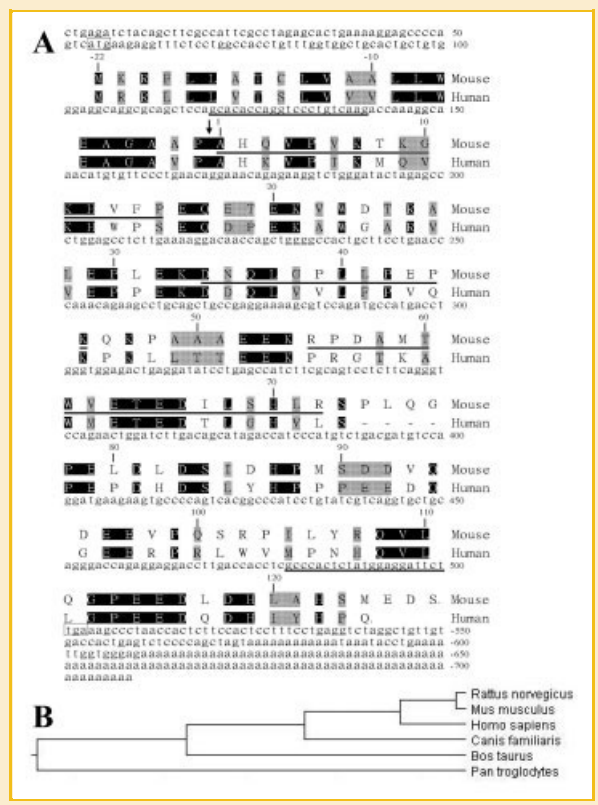

Fig. 2. Protein sequence of mouse PRAP and evolutionary relationship of mammalian PRAPs. A: Nucleotides in the reading frame of *PRAP* cDNA are in lowercase letters. The initial and stop codons are boxed. The oligonucleotides of primer pair used for RT-PCR are underlined. The deduced protein sequences are given in one-letter code numbered from the post-translational cleavage indicated by an arrow. The peptide sequences assigned from direct protein analysis and from MS analysis of the trypsin-digested spot (a) sample are underlined. Sequences of mouse and human PRAPs were aligned. Identical residues are highlighted and similar residues shaded. B: A phylogenetic tree for the mammalian PRAP family was constructed by the neighbor-joining method. Full-length protein sequences of each gene were used for the analysis. The length of each horizontal line is proportional to the substitution frequency.

Antiserum against the recombinant PRAP was raised in New Zealand white rabbits.

Following the method of Towbin et al. [1979], the proteins on the 2D gel were transferred to a PVDF filter. Membranes were immersed in a blocking solution of PBS-0.1% (v/v) Tween-20 (PBST) containing 5.0% (w/v) skim milk for 1 h, followed by another hour of incubation at room temperature with the PRAP antiserum diluted to 1: 10,000 in the blocking solution. After gently agitating in four changes with PBST for 15 min each, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted to 1: 10,000 in the blocking solution for 1 h. Immunoreactive spots were revealed using an enhanced chemiluminescent substrate according to the manufacturer's instructions.

EXAMINATION OF PRAP ENDOCYTOSIS

The recombinant PRAP was labeled using Alexa Fluor® 488 Protein Labeling Kit (Invitrogen, Carlsbad, CA). The modified derivative was designated as AF488-PRAP. Ishikawa cells (2×10^5) seeded on a coverslip in a plastic well were cultured at 37°C for 24 h, washed twice with PBS, and incubated in phenol red-depleted MEM containing 1.0 μM AF488-PRAP alone or together with 10-fold excess of unlabeled PRAP, 5.0 μg/ml of a fluorescent endosome marker *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)-phenyl)hexatrienyl)pyridinium dibromide (FM4-64, Invitrogen, Carlsbad, CA), or 50 nM of a fluorescent lysosome marker [N-[2-(dimethylamino)ethyl]-5-[[5-(1H-pyrrol-2-yl)-2H-pyrrol-2-ylidene-kN]methyl]-1H-pyrrole-2-propanamidato-kN1] difluoro-Boron (LysoTracker® Red DND-99, Invitrogen, Carlsbad, CA) at 37°C for 60 min. Cells were washed twice with PBS, exposed to PBS containing 2% formaldehyde, incubated at room temperature for 15 min, washed with PBS again, and stained with DAPI to reveal the nuclear DNA. The fluorescence dots in relation to the nuclear morphology were observed under a Zeiss LSM 510 confocal microscope (Carl Zeiss Microscopy, Jena, Germany).

ALKALINE PHOSPHATASE ACTIVITY

Protein concentrations were determined using the BCA protein assay. Alkaline phosphatase activity in Ishikawa cells was measured using a modified version of the method of Littlefield et al. [1990].

STATISTICAL ANALYSIS

Data are expressed as the mean ± SD of at least three independent experiments. Differences were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test using Instat software (Graph Pad, San Diego, CA). A P-value < 0.05 was considered to be significant.

RESULTS

DEMONSTRATION OF PRAP IN THE ULF OF ADULTS BY PROTEOMIC ANALYSIS

Figure 1A and B respectively display the resolution of ULF proteins from adult mice and immature mice injected with DES (0.1 $\mu g\, g^{-1}\, day^{-1}$) for three consecutive days by 2D gel electrophoresis. Mapping the silver-stained protein spots on the two gel slabs reveals that spots (a) and (b) around 18–20 kDa in Figure 1A, both were isoelectrofocused about pH 4.4–4.6, are absent in Figure 1B. The proteins on Figure 1A were transferred to a PVDF filter. Automated Edman degradation of spot (a) for 15 cycles gave reliable data to establish Ala as the N- terminal residue and the N-terminal sequences of AHQVPVKTKGKHVFP, which completely aligns at every position with the N-terminal region of mouse *PRAP* cDNA-deduced protein sequences (Fig. 2A). The same N-terminal sequences were also determined in spot (b). Further, we produced a recombinant his_6-tagged mouse PRAP in the *E. coli* strain BL21 (DE3), purified it to homogeneity (Fig. S1), and used it to induce the rabbit antiserum. Among the adult ULF proteins, the antiserum only interacted with spots (a) and (b) (Fig. 1C), showing the high specificity of the antiserum. These data together suggest spots (a) and (b) derived from mouse PRAP.

We performed in gel digestion for spot (a) by trypsin and determined the molecular mass of each protease digest by MALDI MS. Two peptides with a ratio of m/z 1,320 and 2,169 were further analyzed using CID MS/MS to establish the peptide sequences DNQLGPLLPEPK (Fig. 3A) and RPDAMTWVETEDILSHLR (Fig. 3B). These two partial sequences are completely matched with ^{34}D-K^{45} and ^{55}R-R^{72} in the deduced PRAP sequences (Fig. 2A), substantiating spot (a) as a mouse PRAP. The post-translational cleavage is apparent at the peptide bond of ^{22}P-A^{23} in the cDNA-deduced

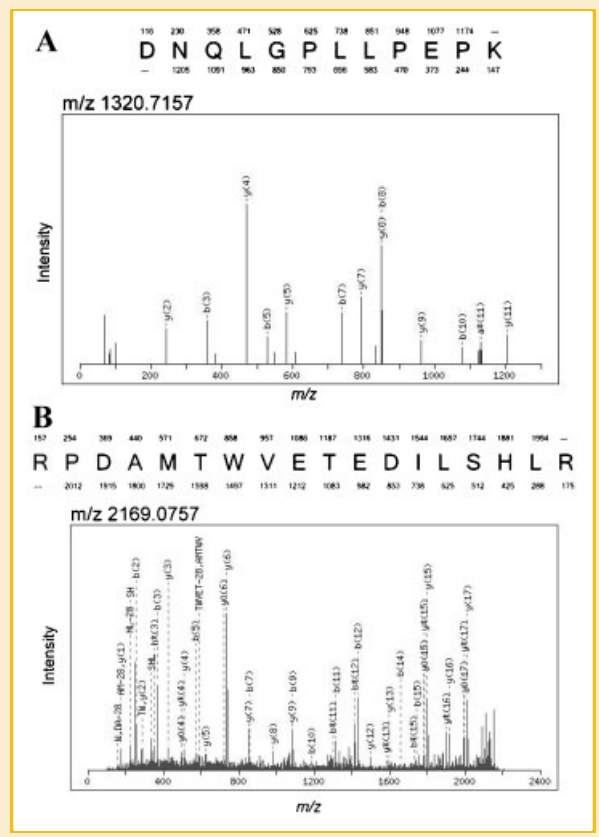

Fig. 3. Partial peptide sequences from MS analysis of spot (a). Two trypsin digests of spot (a) (Fig. 1A) with m/z 1320 and m/z 2169 on the spectral pattern of MALDI-Q-TOF were subjected to a CID MS/MS to establish the peptide sequences shown in (A) and (B). The predicted masses of ion type b and y are shown above and below the peptide, respectively.

protein to yield a mature protein containing 127 amino acid residues and a signal sequence of 22 amino acid residues, which show the general feature of a signal peptide that contains a 7–11-residue hydrophobic core preceded by one or two basic residues at the N-terminus of some eukaryotic secretory proteins. Such a characteristic is consistent with the secretory nature of PRAP.

One representative pattern of phylogenetic tree established for six mammalian PRAPs suggests that they have a common evolutionary origin (Fig. 2B). They can be divided into several subgroups in which human and rodent PRAPs may have duplicated from a more recent progenitor, according to the substitution frequency. These two proteins share 50% identical and 27% similar residues (Fig. 2A), indicative of their high degree of conservation.

EXTRACELLULAR PRAP ENHANCES THE ESTROGEN RESPONSIVENESS OF ISHIKAWA CELL

Ishikawa cells, which are derived from a well-differentiated adenocarcinoma of the human endometrial epithelium, have been used as a surrogate cell model to evaluate the estrogenicity of a drug [Holinka et al., 1986a,b; Albert et al., 1990; Wober et al., 2002]. We cultured the cells in MEM devoid of phenol red and fetal bovine serum at 37°C. The cells remained viable as the incubation time up to 48 h. To assess the internalization capability of PRAP, the cells were incubated with AF488-PRAP (see Materials and Methods section) in PBS at 37°C for 1 h, and the fluorescent signal was traced. The signal appeared in the cytoplasm of almost all portion of cells but was not found as an excess of unlabeled PRAP was added to the cell incubation (cf. a and b of Fig. 4A). Most of the AF488-PRAP signal was distinctive to the signal due to either a lysosome marker (cf. e, f ,and g of Fig. 4A) or an endosome marker (cf. h, i, and j of Fig. 4A). No signal was seen in the cells being incubated with AF488-PRAP and a clathrin-mediated endocytosis inhibitor MDC together (c, Fig. 4A). Nearly no AF488-PRAP signal appeared on the cell membrane and in the cytoplasma of cells incubated at 4°C (d, Fig. 4A), suggesting a weak affinity of PRAP to the cells.

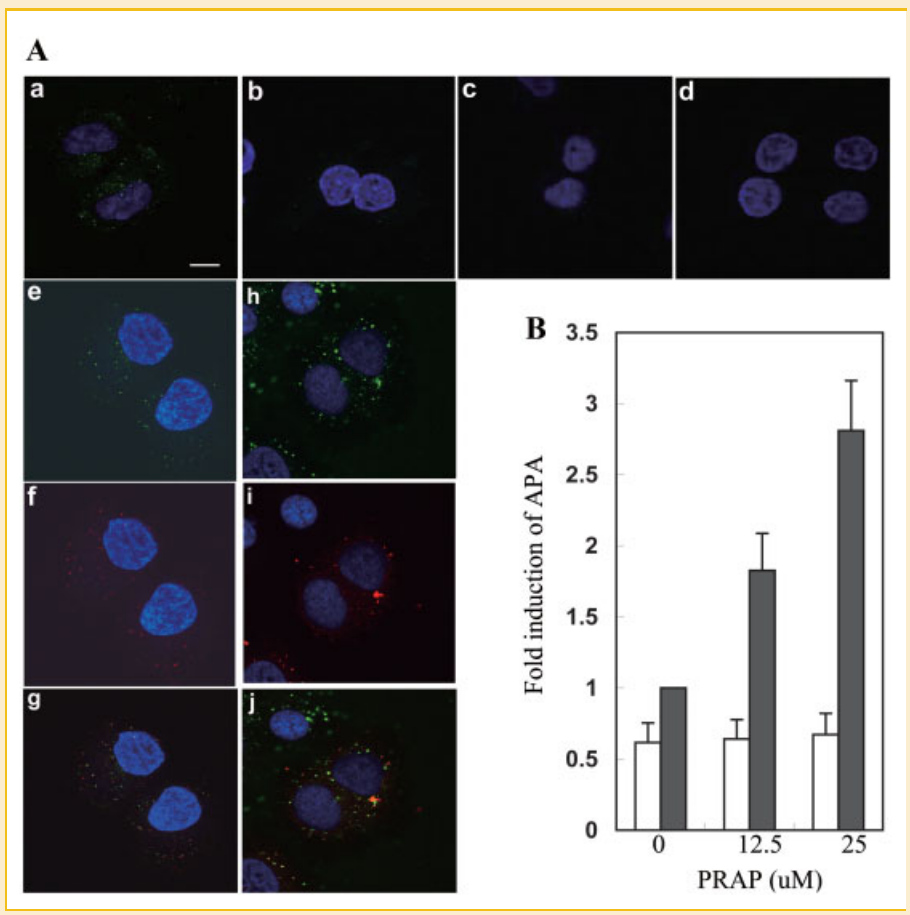

Fig. 4. Exrtracellular PRAP enhances the E_2-stimulated alkaline phosphatase activity of Ishikawa cells. A: Internalization of PRAP. The cells on cover slips were incubated at 37°C for 1 h with AF488-PRAP (1 μM) in MEM alone (a) or in the presence of unlabeled 10 μM PRAP (b), 50 μM MDC (c), 50 nM of a fluorescent lysosome marker (e, f, and g), or 5.0 μg/ml of a fluorescent endosome marker (h, i, and j). In relation to DAPI-stained nucleus (blue color), the green fluorescent dots of AF488-PRAP (a, e, and h) and the red fluorescent dots due to either the lysosome marker (f) or the endosome marker (i) were examined under a confocal microscope; (g), merge of (e) and (f); (j), merge of (h) and (i) (See Materials and Methods section for details). The experiment of (a) was performed at 4°C (d). Scale bar, 10 μm. B: The cells were cultured in phenol red-depleted MEM containing 0–25 μM PRAP alone (open columns) or in the presence of 0.1 nM E_2 (solid columns) for 48 h at 37°C. Equal protein amount from the lystes of each cell treatment were used to determine alkaline phosphatase activity (APA). The reported values, which are expressed as fold induction relative to the enzyme activity of E_2-treated cells in the absence of PRAP, represent the mean ± SD of triplicate measurements from three independent experiments.

Alkaline phosphatase activity is a convenient and sensitive marker of estrogen action in Ishikawa cells [Holinka et al., 1986a; Littlefield et al., 1990]. We measured the enzyme activity in the homogenates of cells being incubated in the culture medium containing 0–25 μM PRAP alone, or in the presence of 0.1 nM E_2 at 37°C for 48 h (Fig. 4B). Relative to the control, E_2 alone stimulated enzyme activity although PRAP alone did not. However, the enzyme activity of E_2-treated cells could be elevated 1.8-fold by 12.5 μM PRAP and 3.0-fold by 25 μM PRAP. These data in line with the results of Figure 4A may suggest rapid entrance of PRAP to enhance the cell estrogen-responsiveness.

Further, the mRNAs of the five estrogen-responsive genes in Ishikawa cells, including *ALPP*, *ALPPL*, *TGF-α*, *PR*, and *Wnt7a* [Leong et al., 2004; Wagner and Lehmann, 2006; Naciff et al., 2009], were amplified from the cell homogenates. E_2 alone increased the transcripts of *ALPP*, *ALPPL*, *TGF-α*, and *PR*, and reduced the transcript of *Wnt7a* as compared to the control (cf. lanes 1 and 3 in each graph of Fig. 5). While 25 μM PRAP alone in the cell incubation had a slight impact on the expression of these five genes (cf. lanes 1 and 2 in each graph of Fig. 5), E_2 together with PRAP greatly increased the E_2-stimulated transcriptions of *ALPP*, *ALPPL*, *TGF-α*, and *PR*, and markedly reduced the E_2-suppressed transcription of *Wnt7*a (cf. lanes 3 and 4 in each graph of Fig. 5). Relative to the transcript level of E_2-treated cells, the transcription of *ALPP*, *ALPPL*, *TGF-α*, or *PR* was up-regulated approximately 5.0-, 2.5-, 3.2-, or 3.3-fold, and *Wnt7*a was down-regulated to around 55% by PRAP. Together, these data manifest the ability of extracellular PRAP to enhance the estrogen-responsiveness of endometrial epithelial cells. Since the estrogen responsiveness of Ishikawa cells occurred unless the cell incubation for more than 24 h, we did not examine whether MDC inhibited the enhancing effect of PRAP on the cellular responsiveness of estrogen, because the cells were not viable when they were incubated with MDC for such a long time.

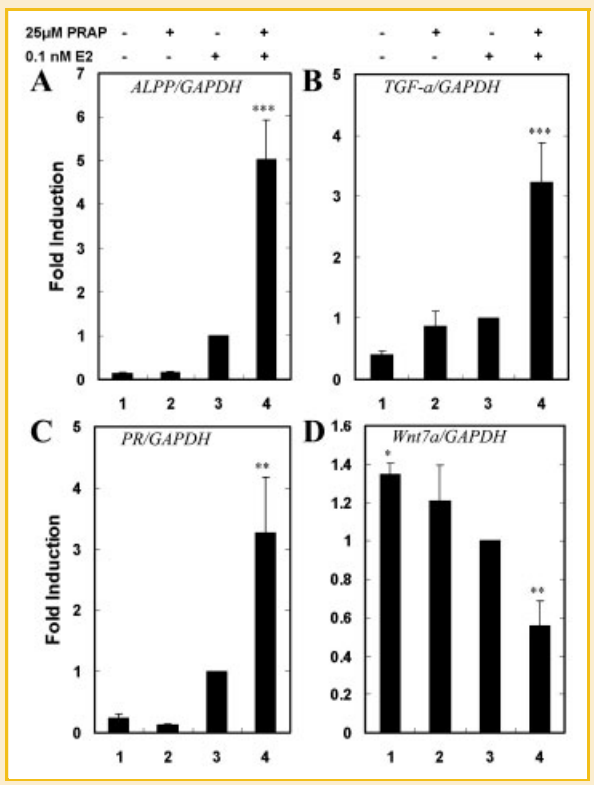

Fig. 5. Extracellular PRAP affects the E_2-responsive gene expression in Ishikawa cells. Similar to the incubation condition described in Fig. 4B, the cells were cultured in the presence of 25 μM PRAP (lane 2), or 0.1 nM E_2 (lane 3), or both together (lane 4). The transcripts of *GAPDH* as the internal control, *ALPP* (A), *TGF-α* (B), *PR* (C), and *Wnt7*a (D) were quantified by Q-PCR from the homogenate of each cell treatment (Materials and Methods section). Each transcript level is represented by a ratio of its cDNA/*GAPDH* cDNA normalized to that obtained from stimulation by E_2 alone. The data are the average of three determinations and the error bars are the SD. The paired statistical comparison by one-way ANOVA: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

DISCUSSION

The presence of PRAP in ULF of estrous female is indicative of its secretory nature (Fig. 1A). This agrees with the characteristic signal peptide in the genomic structure (Fig. 2A). PRAP identified as an 18–20 kDa protein by SDS–PAGE (Fig. 1A) has a molecular weight of 14,600 according to the cDNA-deduced protein sequence. The rather slow electrophoretic mobility may be partially accounted by its acidic property or may reflect some other protein modification, i.e., phosphorylation or glycosylation.

Given that mouse PRAP and human PRAP are highly conserved in the mammalian PRAP family (Fig. 2), our study may be advantageous in understanding the functional role of PRAP in mammalian reproduction. Uterine *PRAP* is expressed under the serum level of ovarian steroids (Fig. 1A). No uterine *PRAP* transcript was found in immature mice, but injection of the animals with E_2 at a dose of 0.1 $\mu g\, g^{-1}\ day^{-1}$ could stimulate the uterine PRAP transcription (unpublished work). DES at the same dose as E_2 did not initiate the uterine *PRAP* transcription (Fig. 1B), despite that DES is much stronger than E_2 with regard to the estrogenic activity. It is well known that precocious exposure to estrogen disrupts normal postnatal reproductive tract development, which must proceed in the absence of high levels of circulating estrogens. DES can stimulate the uterine cell proliferation of immature mice. However, this endocrine disruptor may pose a risk to animal and human health by both adversely affecting reproduction and development and by promoting certain type of tumors. For instance, Yoshida et al. [1999] found abnormal differentiation of endometrial epithelial cells in DES-treated immature mice, and it is well documented that previous clinical use of DES in pregnant women for treatment of abortus imminens results in adverse effect in their progeny health. For the time being, it is unclear whether the reciprocal impact of uterine *PRAP* expression by the high potent estrogenic agent DES contributes to its pathogenic effect.

The *PRAP* transcript has been shown as a useful in vitro marker for the regulation of mammalian epithelial differentiation and intestinal development [Tou et al., 2004; Lepourcelet et al., 2005]. This work is the first one illustrating rapid entrance of exogenous PRAP into Ishikawa cells and its ability to enhance the cell estrogen-responsiveness (Figs. 4 and 5). Our data may lend support to the view

of PRAP as a paracrine regulator for the endometrial estrogen response. It invites to study how estrogen regulates the uterine *PRAP* expression and to assess whether this protein is involved in the uterine remodeling during the reproduction cycle.

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