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## ePAD, an oocyte and early embryo-abundant peptidylarginine deiminase-like protein that localizes to egg cytoplasmic sheets

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### Abstract

Selected for its high relative abundance, a protein spot of MW ~75 kDa, pI 5.5 was cored from a Coomassie-stained two-dimensional gel of proteins from 2850 zona-free metaphase II mouse eggs and analyzed by tandem mass spectrometry (TMS), and novel microsequences were identified that indicated a previously uncharacterized egg protein. A 2.4-kb cDNA was then amplified from a mouse ovarian adapter-ligated cDNA library by RACE-PCR, and a unique 2043-bp open reading frame was defined encoding a 681-amino-acid protein. Comparison of the deduced amino acid sequence with the nonredundant database demonstrated that the protein was ~40% identical to the calcium-dependent peptidylarginine deiminase (PAD) enzyme family. Northern blotting, RT-PCR, and in situ hybridization analyses indicated that the protein was abundantly expressed in the ovary, weakly expressed in the testis, and absent from other tissues. Based on the homology with PADs and its oocyte-abundant expression pattern, the protein was designated ePAD, for egg and embryo-abundant peptidylarginine deiminase-like protein. Anti-recombinant ePAD monospecific antibodies localized the molecule to the cytoplasm of oocytes in primordial, primary, secondary, and Graafian follicles in ovarian sections, while no other ovarian cell type was stained. ePAD was also expressed in the immature oocyte, mature egg, and through the blastocyst stage of embryonic development, where expression levels began to decrease. Immunoelectron microscopy localized ePAD to egg cytoplasmic sheets, a unique keratin-containing intermediate filament structure found only in mammalian eggs and in early embryos, and known to undergo reorganization at critical stages of development. Previous reports that PAD-mediated deimination of epithelial cell keratin results in cytoskeletal remodeling suggest a possible role for ePAD in cytoskeletal reorganization in the egg and early embryo.

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### Introduction

During growth, the oocyte accumulates a pool of maternal gene products and organelles required for early development. In the fully grown egg, the transcriptional machin-

ery is silent and, once ovulated, the terminally differentiated egg will die if it does not bind and fuse with a sperm. If fertilization occurs, however, maternal gene products orchestrate the transformation of the egg into a totipotent zygote within several hours. Following gamete fusion, calcium transients propagated in the egg cytoplasm lead to the activation of signal transduction cascades, which are thought to mediate early embryonic events, such as remodeling of the cortical cytoskeleton, cortical granule exocytosis, completion of meiosis, polar body emission, and for-

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mation of the male and female pronuclei (Yanagimachi, 1994). Reprogramming of the parental chromatin is also thought to occur soon after fertilization, and by the two-cell stage, maternal transcripts begin to be replaced by embryonic transcripts, and the embryonic genome is activated (Schultz et al., 1999). Maternal factors, however, continue to persist in the early embryo until at least the morula stage of development (Pratt et al., 1983).

Many of the structural and molecular mechanisms mediating the physiological changes in the early embryo are as yet incompletely characterized. One of the most abundant cytoskeletal components of the mammalian egg is a fibrous network of intermediate filaments (Gallicano et al., 1994a; Lehtonen, 1985, 1987; Lehtonen et al., 1983; Uranga et al., 1995) named the cytoplasmic sheets (Capco et al., 1993). These organelles were previously thought to be either yolk platelets or possibly ribosome storage sites (Yanagimachi, 1994); however, electron microscopic studies indicate that the highly ordered sheets are composed of parallel arrays of ~10-nm fibers (Gallicano et al., 1991). This filamentous network is stabilized by cross-bridges and is overlain with a tightly packed particulate material. Solubility and immunological studies indicate that the Tween 20 insoluble cross-linked fibers contain keratin (but not vimentin or tubulin) and the soluble fraction largely consists of an unidentified ~69-kDa protein (Capco et al., 1993). Soluble protein kinase C associates with the cytoplasmic sheets, phosphorylates cytokeratin and the ~69-kDa soluble protein, and may be responsible for initiating the changes in spatial organization that these sheets undergo at the time of fertilization (Gallicano and Capco, 1995). Cytoplasmic sheets arise during oocyte development (Gallicano et al., 1994b), are unique to the egg and early embryo, are conserved among mammals (Gallicano et al., 1992), and undergo extensive spatial reorganizations during the critical developmental transitions of fertilization, compaction, and blastulation (Capco and McGaughey, 1986).

Peptidylarginine deiminases (PADs) are a family of calcium-dependent sulphydryl enzymes that convert arginine residues to citrulline in proteins (Senshu, 1990). PAD activity appears to be upregulated by a variety of estrogenic compounds (Nagata et al., 1990; Senshu et al., 1989; Takahara et al., 1992), and to date, four types of PADs have been characterized with each differing in its pattern of substrate specificity and tissue distribution. For example, the widely distributed and well characterized type II PAD is especially abundant in muscle (Takahara et al., 1983, 1986) and brain (Akiyama et al., 1999; Asaga and Ishigami, 2001) and is associated with deimination of myelin basic protein (Lamensa and Moscarello, 1993; Moscarello et al., 2002). PAD V, found in granulocyte-differentiated HL-60 cells, is thought to play a role in myeloid cell differentiation (Nakashima et al., 1999), and likely targets nucleophosmin and histone core proteins for deimination (Hagiwara et al., 2002). Type I and III PADs have been characterized in the epidermis; with type III PAD being found to deiminate

trichohyalin in hair follicles (Kanno et al., 2000; Nishijyo et al., 1997) and Type I PAD deiminating keratin and filaggrin during epidermal differentiation (Akiyama and Senshu, 1999; Senshu et al., 1999a). It is thought that the deimination of keratin and filaggrin in the epidermis induces changes in the spatial organization of keratin intermediate filaments during keratinocyte maturation (Ishida-Yamamoto et al., 2002). Deiminated keratin has been identified in day 18 embryos (Akiyama and Senshu, 1999); however, the presence of deiminated keratin at earlier stages of development has not been investigated.

In an ongoing project to identify previously uncharacterized proteins in the ovulated mouse egg, we have been performing tandem mass spectroscopic analysis of abundant oocyte proteins that have been cored from Coomassie-stained two-dimensional (2D) gels. One dominant egg protein spot that yielded novel peptide microsequences was cloned and the cDNA characterized. Using Northern blotting, the tissue distribution was determined and the protein was found to be expressed in primary oocytes and to persist until at least the blastocyst stage of development. Due to its 40% identity with the peptidylarginine deiminase enzyme family, the name ePAD, for egg and embryo abundant PAD, was selected. Remarkably, at the ultrastructural level, ePAD localized to the egg's cytoplasmic sheets, a cytoskeletal structure unique to the egg and early embryo. The discovery that ePAD is associated with the egg cytoplasmic sheets leads to the hypothesis that arginine deiminase reactions directed against cytokeratin, and possibly other proteins, results in reorganization of the cytoskeleton during early development.

## Materials and methods

### *Two-dimensional electrophoresis*

Mouse oocytes (2850 for the Coomassie-stained gel in Fig. 1 and ~300 per blot in Fig. 6) were collected and dezonulated as described previously (Coonrod et al., 1999). The zona-free eggs were then washed six times in PBS containing 10 µg/ml polyvinylalcohol (PVA; Sigma) and extracted in Celis lysis buffer [containing 2% (v:v) NP-40, 9.8 M urea, 100 mM dithiothreitol (DTT), 2% ampholines (pH 3.5–10), and protease inhibitors] for 30 min at room temperature (Rasmussen et al., 1991). Isoelectric focusing (IEF) was performed by using the BioRad Protean II Multi-Cell apparatus with an ampholine mixture (Pharmacia Biotech, Uppsala, Sweden) of pH 3.5–5 (30%), 3.5–10 (40%), 5–7 (20%), and 7–9 (10%). The tube gels were placed on 12% slab gels (16 × 16 cm, plates 1.5 mm diameter), and the focused proteins were separated in the second dimension. The gels were then either stained with Coomassie or electroblotted to nitrocellulose membranes. For Coomassie staining, the gels were fixed overnight in a solution of 50% ethanol and 10% acetic acid and placed in a solution con-

taining 0.1% Coomassie R250, 40% methanol, and 0.1% acetic acid for 4 h. The gels were then destained in a solution of 10% acetic acid and 50% methanol.

#### Tandem mass spectroscopic analysis of egg peptides

Two ~75-kDa (*pI* 5.5) Coomassie-stained protein spots were cored from the 2D SDS-PAGE gel, fragmented, destained in methanol, reduced in 10 mM dithiothreitol, and alkylated in 50 mM iodoacetamide in 0.1 M ammonium bicarbonate. The gel pieces were then incubated with 12.5 ng/ml trypsin in 50 mM ammonium bicarbonate overnight at 37°C. Peptides were extracted from the gel pieces in 50% acetonitrile and 5% formic acid and microsequenced by tandem mass spectrometry at the Biomolecular Research Facility of the University of Virginia.

#### Adapter-ligated cDNA library construction and RACE-PCR cloning of ePAD cDNA

Two micrograms of mouse ovarian poly(A)<sup>+</sup> mRNA, isolated using the FastTrack 2.0 kit from Invitrogen (Carlsbad, CA), was used as the template for the construction of a Marathon adaptor ligated cDNA library (Clontech, Palo Alto, CA). Oligo(dT) primers, as well as avian myeloblastosis virus (AMV) reverse transcriptase, were used to construct the first strand of cDNA. The RNA was digested and the second strand of cDNA was synthesized in a simultaneous reaction with a mixture of *Escherichia coli* DNA polymerase I, RNase H, and *E. coli* DNA ligase. The cDNA ends were then blunted by using T4 DNA polymerase, and Marathon cDNA adaptors were ligated to both ends of the cDNA by the addition of T4 DNA ligase.

RACE PCRs were performed using the AmpliTaq Gold DNA polymerase from Perkin-Elmer (Norwalk, CT) with ovarian library cDNA templates. Cycling parameters were: 94°C, 10 min; 94°C, 15 s; 60°C, 30 s; 72°C, 2 min; and 72°C, 10 min, for 40 cycles. PCR of plasmid templates was performed under the same conditions, except that Promega Advantage 2 taq polymerase was used. The primers for cloning the full-length ORF of ePAD into the TOPO cloning vector (Invitrogen) were 5'-GTACTCGAGATGGTAG-GCATGGAAATCACC-3' and 5'-CGATCTAGAT-GGGTCATCTCCACCACTT-3', and the AP1 primer supplied in the Marathon ready cDNA kit (Clontech).

#### Northern blot analysis

A randomly primed probe was generated corresponding to the 1100-bp N-terminal region of ePAD by using the Prime-a-Gene Labeling System (Promega, Madison, WI). Twenty-five nanograms of cDNA were denatured at 95°C for 5 min. After cooling on ice for 5 min, 50 mM Tris-HCl, pH 8.0, 5 mM MgCl<sub>2</sub>, 2 mM DTT, 200 mM Hepes, pH 6.6, random hexadeoxyribonucleotides, 400 µg/ml of BSA, 333 nM [ $\alpha$ -<sup>32</sup>P]dCTP, 20 µM each of dATP, dGTP, and dTTP,

and 5 units of DNA polymerase I, large (Klenow) fragment, were added to the cDNA. This was incubated for 2 h at 22°C, and subsequently passed through a NucTrap (Stratagene, La Jolla, CA) column to remove unincorporated nucleotides from the probe. A mouse multi-tissue Northern blot (Clontech) was incubated with ExpressHyb hybridization solution (Clontech) for 1 h at 65°C. The probe was heated to 95°C for 5 min, and placed in 10 ml of fresh ExpressHyb. This was incubated with the blot overnight at 55°C, and washed on the following day, twice for 15 min with 2× SSC and 0.5% SDS, twice for 30 min in 0.1× SSC and 0.5% SDS at 65°C. Signals were detected by exposure to X-ray film for 24 h and for 10 days.

#### RT-PCR of ePAD in mouse tissues

The Mouse Multiple Tissue cDNA Panel from Clontech, comprised of cDNA from 11 different tissues, served as template in the PCRs. cDNAs from the Panel were used to amplify ePAD and control G3PDH cDNA fragments in 50-µl reactions (10 mM Tris-HCl, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1% Triton X-100, 0.2 mM of each dNTP, 0.5 U of Taq polymerase, and 0.5 mM of each primer). Thermocycler settings consisted of a 10-min incubation at 95°C, followed by 40 cycles at 95°C (15 s), 60°C (30 s), and 72°C (2 min). Extension progressed for 10 min at 72°C. Ten percent of product was later analyzed on 1.5% agarose gels and confirmed by Southern blotting. The following ePAD primers were used: 1–27, 5'-GGTAAGGACTGCTGACAGTGGC-TAGCT-3'; 2222–2249, 5'-CCTCCAGAAG-TGTCAAGCTGGTAGATGT-3'. G3PDH primers were included in the previously mentioned Clontech kit.

#### In situ hybridization

Ovaries from fertile ICR female mice were fixed in neutral buffered formalin solution (4%) (Sigma, St. Louis, MO) for 12 h. Following dehydration, the blocks were embedded in paraffin, sectioned (2.5 mm), and mounted on positively charged slides. To prepare a riboprobe, a DNA fragment consisting of the 5' 250 nucleotides of the ePAD ORF was subcloned into the pBluescript SK plasmid vector and used as template for in vitro transcription (Angerer et al., 1987). Tritiated Q2 uridine triphosphate (UTP) was incorporated into the riboprobes by either T3 (sense) or T7 (antisense) RNA polymerase. A labeled β-actin riboprobe was used as a positive control. The sections were deparaffinized, rehydrated, and treated with proteinase K. The in situ hybridization solution contained 50% formamide, 0.3 M NaCl, 20 mM Tris-HCl, 1 mM EDTA (pH 8.0), Denhardt's solution, 500 mg/ml yeast tRNA, and 10% dextran sulfate. The final probe concentration was normalized for probe length and applied at full saturation (0.2 mg/ml/kb complexity). The hybridization was carried out at 55–65°C. After hybridization, the sections were washed under high-stringency conditions to remove nonspecific hybridization.

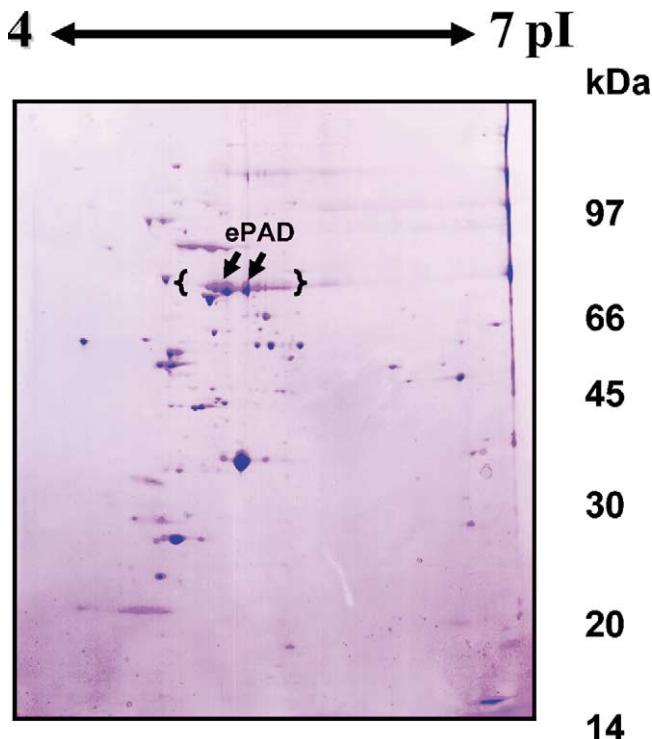


Fig. 1. Location of ePAD on a Coomassie-stained 2D electrophoretic gel of 2850 NP-40 extracted zona-free mouse oocytes. Arrows indicate the two protein spots that were cored from the gel for TMS analysis. The putative ePAD protein chain is delineated by the brackets.

The slides were overlaid with autoradiography emulsion, exposed 2–4 weeks at 48°C, developed photographically, and lightly stained with hematoxylin and eosin. Dark field images were then captured at 200× by using a Zeiss microscope.

#### *Expression of recombinant protein and antibody production*

Primers were designed to generate a PCR product encoding amino acids 1–200 of the open reading frame. The sense primer, 5'-TAAGGATCCGATGGTAGGCATGGAAATCACCTTGG-3', contained an engineered *Bam*H I restriction site, while the anti-sense primer, 5'-GTACTCGAGCCAGCCTCTGAGACCAGTAGACTCT-3', had a *Xho*I restriction site. The primers were used to amplify the cDNA fragment, and the PCR product was cloned into the *Bam*H I–*Xho*I sites of the pET22b expression vector (Novagen, Madison, WI). Once the construct was verified, it was transformed into *E. coli* strain BL21 DE3 cells (Stratagene). A single positive colony was used to inoculate a 10-L culture that was grown at 37°C in Luria broth (LB), in the presence of 50 mg/mL ampicillin until the A600 reached 0.6. Recombinant protein expression was induced with 1.0 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 3 h.

Protein was purified from the cells by Ni-affinity chromatography, which binds to a 6× His tag attached to the recombinant protein (Reddi et al., 1994). Half of the biomass was suspended in 5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9, and was sonicated four times for 45 s. The sonicated extract was centrifuged at 20,000g for 15 min to collect inclusion bodies and cellular debris. The supernatant was removed, and the pellet was then resuspended in 6 M urea, 5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9. This was incubated on ice for 1 h to completely dissolve the protein. The remaining insoluble material was removed by centrifugation at 39,000g for 20 min and filtration through a 0.45-micron membrane. The remaining supernatant was bound overnight at 4°C to nickel charged by His-Bind Resin (Novagen) while shaking gently. After centrifugation, the resin was washed twice for 20 min with 5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9, and once with 20 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9, for 20 min. The product was eluted with 1 M imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9, for 20 min and collected. Elute from the column was passed through a PREP cell (BioRad), in order to remove remaining contaminants. Final product was then dialyzed against 1.0 mM ammonium bicarbonate for 36 h before lyophilization and reconstitution.

#### *Polyclonal antibody production and IgG purification*

Three adult male Hartley guinea pigs (albino retired breeders) were used for antibody production against a partial fragment (extreme 200 N-terminal amino acids) encoded in the pET22b/ePAD clone. Preimmune serum was collected by heart puncture, and subsequently, each animal was injected with 100 µg of purified recombinant PAD (rePAD) in an emulsion with Freund's complete adjuvant. Each animal received two booster immunizations with 50 µg of purified rePAD in Freund's incomplete adjuvant at 3-week intervals. For all immunizations, half of the antigen emulsion was injected intramuscularly in the leg and half subcutaneously in three sites on the back. All animals were then exsanguinated by heart puncture 10 days after the final immunization, and the blood was collected in serum separation tubes (Becton Dickinson, Franklin Lakes, NJ). After centrifugation at 1750g for 10 min, the serum was removed and frozen until needed. A prepak Protein A column from Pierce was used for purifying IgGs from anti-rePAD mouse serum. The column was prewashed with 5 mL of sterile water. To calibrate the column, 5 mL of binding buffer supplied by Pierce was then added and allowed to drain. Sera was diluted 1:10 with binding buffer, passed through the column in 4-mL increments, and the bound resin was washed with binding buffer. IgGs were eluted from the column with elution buffer from Pierce and dialyzed against PBS before quantitating.

gggttaaggactgctgacagtggtagttggtaagcccaagccatgtctttcagaactca  
 M S F Q N S  
 CTCAGCTGTCCTGGTCAATCCACCATGCCCTGTGCATGGTAGGCATGAAATCACC  
 L S L S L V N P T H A L C M V G M E I T  
TTGGACATCAGCAACTGTGACCCGGACAAGTCAACTCTTCACCATCGTGTTCCCCC  
 L D I S K C A P D K C K S F T I R G S P  
AGGATCTTGATCCACATCTACCTCCGTACCGTGGCAAAGGAGGACTGTGGCTGG  
 R I L I H I S S S V I A G K E D T V V W  
AGGTCATGAAACATCCACAGTGGTAGGTGGAGATGGTGGGCCAGCCCCACTGTG  
 R S M N H P T V A L V R M V A P S P T V  
GATGAAGACAGGTGCTGGTCTCTACTCTGTCCCTGACCAAGAAGTCCCCCAGGG  
 D E D K V L C Y F C P D Q E V P T A T  
GCTGTGCTGTTCTCACCGCATCGAGATCTCCCTGGAGGAGACATCTACGAGATGGA  
 A V L F L T G I E I S L E A D I Y R D G  
CAACTGACATGCAAGTGATAAGCAAGCTAAGAAAAATGGATGTGGGTATGAAACGGC  
 Q L D M P S D K Q A K K K W M W G M N  
TGGGGAGCCATCTGTGTAATTGTAGGCCATTAGCTGTGGCCAGCCTGTGAAACAG  
 W G A I L L V N C S P N A V Q G P D E Q  
TCCCTTAGGGGGCCAGAGAACATAGAACACAACCTGTCTCATGATGTAACGTG  
 S F Q E G P R E I Q N N L S Q M N V T V  
GAGGGCCCAACAGCATCTACAGAAATTACAGATTGATCTACATACCTCCGAAGAAGAG  
 E G P T S I N Q Y Q L I L H T S E E E  
GCGAAGAACAGAACAGAGTCTACTGGTCTCAGAGGCTCTGTGATGAACTGGTGGT  
 A K K T R V Y W S Q R G S S A Y E L V V  
GGACACCAACAGGCCATCTCTCTGGCTACCTTGAGAACGCTAGGAAAGGGCTTC  
 G P N K P V Y L L P T F E N R K E A F  
TACGTAGAACGGCACGGAATTCCCATCTCCAGCTCTCGGGCTGTATCTTGTCACTC  
 Y V E A T E F P S P S F S G L I S L L  
TCCCTAGTAGAAAAGGCTCACGACGAGTGCATCCAGAGATTCGGCTATAAGGATACA  
 S L V E K A H D E C I P E I P L Y K D T  
GTGATGTTCCGGTGGCACCTATCTCATGCCAGCACCAGATGCCCTAGAGGTT  
 V M F V A P Y I F M P S T Q M P L E V  
TACCTGTGCAAGGAGCTACAGTCAGGCTTGTGGACTCAGTGACCAAGCTGAGCGAG  
 Y L C R E L Q L Q G F E D S V T K L S E  
AAGAGCAAAGTCAGGTGTAAGGTCTATGAGGACCCAACCGCAGAGCAAGTGGCTC  
 K S K V Q V V K Y E D P N R Q S K W L  
CAGGAGAGATGGCTTCTGCTACTACAGGCTCTCACAGGGCTGTGATCCTGGCTT  
 Q D E M A F C Y T Q A P H K T V S L I L  
GACACCCCAAGGGTCCAAGCTGGAAGACTTCCCATGAAATACACACTGACCCCTGGC  
 D T P R V S K L E D F P M K Y T L T P G  
TCTGGTACCTGTACCGAACAAATTGAGGACCCGGGTGGCTGGCTGGATTCCATGGG  
 S G Y L L I R Q I E D H R V A S L D S I G  
AACTGTGATGGTATCTCCGCCGTCAAGGCTCAGGGCAAAAGACTACCCCTAGGGGTC  
 N L M V S P P V K A Q G K D Y P L G R V  
CTCATTGGTGGCAGCTTACCCAGCTCTGGGGCCGGACATGAACAAGGGCTCGCA  
 L I G G S F Y P S S E G R D M N K G L R  
GAATTCTGTATGCCAGGGTGTGAGGCCCCCTGTGGAACCTCTCTCGGACTGGCTGATG  
 E F V Y A Q Q V Q A P P V E L F S D W L M  
ACCGGTACATGGATCAATTCTATGTTGCTTGTCCCTACCAATGATAAAACACGACCAG  
 T G H M D Q F M C F V P T N D K N N D Q  
AAAGACTCCGCCGTGCTGGCAGCCCACTGGCTGCTTGGACTGTTGAGCTGGAGAAG  
 K D F R L L L A S P S A C F E L F E Q K  
CAGAAGGAAGGCTATGGAACGTGACCCCTGGTGAAGACATTGGAGCAGAACAGCT  
 Q K E G Y G N V T L F E D I G A E Q L L  
TCTAATGGGAGGAGGAGCAAATATTCCCAATCTGGCTGAGAACAGGTTGGAGAG  
 S N G R E S K T I S Q I L A D K S F R E  
CAAGAACACCTATGGAGAAGTGTACGGCTGAACGGCACCCCTGGAGACAGAACAG  
 Q N T Y V E K C I S L N R T L L K T E L  
GGATTGGAGGACAAGGACATCATCTGTACCTCCGCAGCTCTCTGGAGCAGCTGACG  
 G L E D K D I I L I P Q L F C L E Q L T  
AATGTCCTCCCAACCAGCAGACCAAAACTCTCGCAGGGCTACTTCCCGACATG  
 N V P S N Q O S T K L F A R P Y F P D M  
CTGCAGATAATGTTGGCAAGAACCTTGGAACTCCCAAGGCCCTGGGCCAAATC  
 L Q I V L G K N L G I P K P F G P K I  
AATGGCACCTGCTGCCCTAGAAGAGAAAGTGTGGATTACTGGAGGCCCTGGCTCAAG  
 N G T C C L E E K V C G L L E P L G L K  
TGCACCTTCATTGATGATTGACTGCTACCTGGCAACATAGGGAGCTCTGGCAGT  
 C T F D D F D C Y L A N I G D V C A S  
GCCATCATAAACAGGGCCTGGCTGATCAAGTGGTGGAGAGTACCCAtaaacccct  
 A I I N R V P F A F K W W K M T P -  
ggccctggcacggcggcggcggcggcggcggcggcggcggcggcggcggcggcggcgg  
ggcggcggcggcggcggcggcggcggcggcggcggcggcggcggcggcggcggcggcgg  
atcttccatggacttcggacttcggcgggggggggggggggggggggggggggggggggg  
gccccatagggccatggatggatggatggatggatggatggatggatggatggatggat

Fig. 2. Complementary DNA and deduced amino acid sequence of ePAD. The untranslated flanking regions are shown in lower case letters. Peptide sequences originally obtained by mass spectrometry are underlined.

#### Anti-ePAD antibody specificity and ePAD solubility characteristics

BL21 cells expressing rePAD were sonicated and 20 µg bacterial protein, 50 oocytes, and one million sperm per lane

were solubilized in Laemmli buffer (Laemmli, 1970) and heat denatured at 95°C. The samples were then loaded onto a 12.5% linear gel, and separated at 100 V for 3 h. Proteins then electrotransferred onto 0.2 µm nitrocellulose (Bio-Rad Laboratories, Hercules, CA) for 40 min at 100 V for Western blotting.

To investigate solubility characteristics, 70 mouse eggs were extracted with NP-40 lysis buffer (150 mM sodium chloride, 1.0% NP-40, and 50 mM Tris, pH 8.0), incubated on ice for 30 min, and passed through a 25-G needle three times. The extract was then centrifuged for ten minutes at 10,000g. The supernatant and pellet were each solubilized in Laemmli buffer, heat denatured at 95°C, and separated on a 12.5% SDS-PAGE gel under reducing and nonreducing (lacking β-mercaptoethanol) conditions. Protein was then transferred onto a nitrocellulose and blotted as described below.

#### Western blot analysis

For Western blot analysis of ovarian ePAD expression, ovaries from 2, 4, 8, 15 wk, and retired breeder females were homogenized in extraction buffer (PBS, 1% NP-40, and 1× concentration of Complete, EDTA-free Protease Inhibitor Cocktail Table; Roche, Mannheim, Germany). After homogenization, the samples were sonicated for 30 s each and quantified using the Biorad DC Protein Assay kit (Biorad). Equal amounts of each sample (21.5 µg) were loaded on a 12.5% SDS-PAGE gel, and separated at 115 V. Protein was then blotted to nitrocellulose, and stained with Ponceau.

All blots were blocked with 5% nonfat dry milk in PBS with 0.05% Tween 20 (PBS-T) for 30 min, washed and incubated with a 1:5000 dilution of anti-rePAD guinea pig sera. The blots were then washed two times for 10 min in PBS-T, and incubated with a 1:10,000 dilution of peroxidase conjugated goat anti-guinea pig IgG (Jackson ImmunoResearch, West Grove, PA) secondary antibody for 1 h and washed two times for 10 min in PBS-T. The blots were then either developed in TMB peroxidase substrate (3,3',5,5'-tetramethylbenzidine; Kirkegaard and Perry Laboratories, Gaithersburg, MD) or with ECL reagent (Amersham Corp., Buckinghamshire, UK) and developed as described previously (Coonrod et al., 1999).

#### Indirect immunofluorescence of ovarian sections, ovaries, and embryos

Cryosections (5 µm) of mouse ovary were processed for indirect immunofluorescence. Slides were immersed in 95% ethanol for 10 min, washed, and incubated in a blocking buffer of PBS which contained 3% BSA and a 1:50 dilution of normal goat serum for 20 min at room temperature. Next, the slides were incubated with blocking buffer which contained either a 40-µg/ml ePAD IgG or preimmune IgG for 1 h at room temperature, washed, incubated in blocking

PAD type I	1	-----MASPRAVQLSLRKPTTHAVCVVGVEVTLNVYSDVPKCAKTFGVSGSSEVKIYMVY-DPSRVAE	61
PAD type II	1	MQPPIRENLRLERTVRLQYGSRVEAVVVLGTLWDDVYSAAPACAKTFSLKHEGVKVEVVRDGEAEVV	70
PAD type III	1	-----MSLQRIVRVSLLEHTSAVCVAGVETIVDIYGSVPECTDMFEVYGTGPVDIYLSP-SMERNRE	61
PAD type IV	1	-----MAQGAVIHVAPEQPTHAVCVVGTATPLDVRSAPKCYTTFSITASPGVIVDVIH-GPPVKKS	61
ePAD	1	-----MSFQN--SLSLVNPTHACMVGMEITIDISKCAPDKCKSFTIRGSPRLHISS--SVIAG	57
PAD type I	62	PAGWAHWP-IDANVDDVVVVAADTVSKDLYDFKVKSYESQEEAAALAHSVLYLAADVSLSDVDTGRTGKV	130
PAD type II	71	TNGKQRWA-LSPSSTLRLMSAQASTEASSDKVTWVYEEEGSAPIDQAGIFLTIALEISLDVADRDGEVE	139
PAD type III	62	RADTRRWIC-FNKGLEIIIVVMNSPNSNDLNDSHVQIAHYSSREHLPLAYAVIYLNCVDITLDCOMNCADRQD	130
PAD type IV	62	TMGASKWP-IDPELEVTLQVKAASSRTDQKVRSVSYGPKTSP--VQALIYTIGVELSLSADVTRTGRVK	128
ePAD	58	KEDTVVWRSMNHPTVALVRMVASPTVDEDKVLVSYFCPDQEVPATAVIFLTGIEISLEADIYRDGQD	127
PAD type I	131	KGSGDK--KTIWRWGPCCGSGAIIILVNCDR-IHGSREDLHANHLSIEDLQDMSEMLSCGGDELFEHK	197
PAD type II	140	KNNPKK--ASWVWGPEGOGAIIILVNCDRDPWLPKEDCSDEKVYSKQDLODMSOMILRTKGEDRPLPAGE	207
PAD type III	131	RSEFVDK--RQWVWGPDGVGAIIILVNCDRDNVDSNAQDNCQYVRCLQDLODMSVMVIRTOGEALFEDHR	198
PAD type IV	129	PAPAGKDQSTWIWGPCCRGAIIILVNCDKEDPQASGMFEDDKLIDNNDLQDMSEMLSTKTQDKDFFEKYQ	198
ePAD	128	MPSDKQAKKKWWGMNGAIIILVNCSNAVQGPDEQSFGQEPREIQN--NISOMNVTVEGETSILQNYQ	195
PAD type I	198	IVIKASLSDSRRLLKVFCCARG-GTSLSNYKQVLGPRHSSYEVERHSGERAIQYVEGLAFPDASFSGLLSL	266
PAD type II	208	IIVYISMDSDKVGFVYVEN-PFFGQRYIHLGRQKLHYHVVKYTGSAAELLEFVEGLCFPDESFGLVSL	276
PAD type III	199	IVIHTSSCDAERARARVFHVCPEDSCEAVKCVLGPDORMSYEVPLKG--YEERFVEGLSFPDAGFPGLISF	267
PAD type IV	199	IVLEVPKAKMNKVRVFRATR-GKLLSRVYKVALGPQQFSYCLELPGQHSTDYVEGLAFPDADFKGLIPL	267
ePAD	196	IVIHTSEEAKKTRVYWSQR--GSSAYELVVGPNKPVYLLPTFENRRKEAEVATEFPSPSFGLISL	262
PAD type I	267	SVSLVDTTR---PLSEVSVETDSVTFRVAFWIMTPNTCPPIEIVC5VTDIHGRNDKFLEDMSHIAATKANC	333
PAD type II	277	HVSILLEYMA-EGIPLTPIPIDTVMFRAFWIMTPNLIIPPVSVEVCMK---DNYLFLEVKKNLVEKTNC	341
PAD type III	268	HVILLDDSN-EDFSETPIEIDTAVFRVAFWIMTPSTIIPPLEVYVCRVR---NNTCFVEAEEILARKACC	332
PAD type IV	268	TISLSDKSN-PELPEALIVEQDSVTFRVAFWIMTPNTOPPOEVYVCRVS---DNEFLKSLATLTKKAKC	332
ePAD	263	SISLVEKAHDCEITPEIPLYKDITVFMFRVAFVIEMPSTOMPLEVYIREL---QLQGFVDSVTKLSEKSKV	328
PAD type I	334	KLUVCPRAPNNDRWIQDIEFGYIIPAPHKSFPPVSDSPRNRCIIFDFAIKRILGDFGYVTRIEIEFAGAS	403
PAD type II	342	EUKVCFOYMNRCDRWIQDIEFGYIIPAPHKGPFPVVDSPRDGNKDFPIKQILGDFGYVTRPLFETVT	411
PAD type III	333	KLTICPQAENRNDRWIQDMEIGYVCAHKTLIPVPPBDSPRNGELOQFPYKRIILGDFGYVTREPKDSSVS	402
PAD type IV	333	KLTVCPEEIIDDDWQDMEIGYIICAPHKTLIPVPPBDSPRDRGKDFPVKRVMGPNFGYVTRKLYMSELT	402
ePAD	329	QVVKVYEDPNRQSKWQDMEAFCYTCOAPHKTVSLLIDTPRVSKLDFPMKYIITPGSGYLIQIEDHRVA	398
PAD type I	404	GLDSFGNLDVSPSPVVRVGNTDYLPLGRILIG-GSFPKPSGRRMARVVRDFLQAQVQSPVETVSDWLISVGHV	472
PAD type II	412	SLDSFGNLDVSPSPVVTNCGKEYPLGRILIG-SSEPLSCCRMMTKVVRDFLQAQVQAPVETVSDWLISVGHV	480
PAD type III	403	GLDSFGNLDVSPSPVVTNCGKEYPLGRILIG-GNLPGSFGRRVTQVVRNFLHAQKVOPLVETVSDWLISVGHV	471
PAD type IV	403	GLDGFGNLDVSPSPVVTNCGKEYPLGRILIGNSGYSSESRDHQALQDFLISAQVQAPVRLFSDWLISVGHV	472
ePAD	399	SLDSIGNLAVSPSPVKAQGKDYPLGRVLLIGGSFYPSSCGRDMNKGLREFVYAAQVQAPVETVSDWLISVGHV	468
PAD type I	473	DEELSFVPTSDQ---KCFRLLLASPACIOLFQKEEKGYGEAEQFDGLKH---KAKR--SINDIIA	531
PAD type II	481	DEEMTFIPIPGK---KCFRLLLMASTCACYQLEFREKQKAGHGEAVMFKGLGMM---SSKRI--TINKIIS	541
PAD type III	472	DEELSFVPAFDG---KCFRLLLASPACERLFCOKKWKGHGRSLIFEGVIG---DRRVQTVSINOIIIN	533
PAD type IV	473	DEELSFVPEVRDK---QCFRLLLSSPRACYOLFQIISQGHGEATLFEGLK---RKRQ--TINEIIS	530
ePAD	469	DQRMCFVPTNDKNDOKDFRLLLASPACAEELFECKQKEGYGNVNTIFEDIGAEQLISNGRESKTIISQIIA	538
PAD type I	532	DKHJRRDASHVQKCIDWNHREVLIKRELGIISEDIIDIPQLFFLKGA-----YAFAFFPDVMNVV	589
PAD type II	542	NESLTQENQYFORCIDWNHREVLIKRELGIIDIPQLFKMDEN-----HQARAFFPNMVNM	600
PAD type III	534	NQSLINENKFQSCIDWNHREVLIKRELGIIDIPQLFKTEK-----KAVAFFPDIVNML	591
PAD type IV	531	NKKJRDONAYVESCIDWNHREVLIKRELGIIDIPQLFKLVGNS-----RGNSKAOAFFPNMVNM	593
ePAD	539	DKSFREQNTYVEKCISIINRNLILKTELGLIEDKDIIIDIPQLFCLEQLTNVPSNQQSTKLFARPYFPDMQII	608
PAD type I	590	VLGKYLGIKPKPFGLINGCCLEEVRSALLEPLGLFRCVIFDDFLFYHOLLGEIHCGTNVRRPFTFKWW	659
PAD type II	601	VLGKDLGIKPKPFGLPQVEEFCCLETHVRCLEPLGLFCTFIDDLISAYHKFLGEVHCGTNVRRPFAFKWW	670
PAD type III	592	VLGKHLGIKPKPFGLPIINGCCLEEVRSALLEPLGLFCTFIDDLFTEYHMLHGEVHCGTNVRRPFAFKWW	661
PAD type IV	594	VLGKYLGIKPKPFGLPIIDGRCCLEEVRSALLEPLGLFCTFIDDLFTEYHMLHGEVHCGTNVRRPFTFKWW	663
ePAD	609	VLGKNLGIKPKPFGLPKingACCLEEKVCCLEPLGLFCTFIDDLFDCYLANIGDVCASAIINRVPEAFKWW	678
PAD type I	660	SVP 662	
PAD type II	671	MVP 673	
PAD type III	662	MVP 664	
PAD type IV	664	MVP 666	
ePAD	679	MTP 681	

Fig. 3. Amino acid sequence comparison of ePAD with the four known mouse peptidylarginine deiminases (PADs). The sequences were aligned by using the ClustalW alignment program in BioEdit sequence alignment editor. Identical residues are shown in black boxes, and conserved residues are shown in gray boxes. Dashes indicate gaps inserted to maximize sequence alignment. PAD Accession Nos. are as follows: type I, NP\_035198; type II, NP\_032838; type III, NP\_035190 type IV, NP\_035191; ePAD, NP\_694746. PADs I–IV are approximately 50% identical to each other, while ePAD is approximately 40% identical to known murine PADs.

buffer containing goat anti-guinea pig IgG-FITC (1:200 dilution), washed again, and the slides were coverslipped and stored at 4°C prior to imaging. All oocytes and embryos

were obtained from ICR 25–30 g females (Hilltop Labs). Germinal vesicle oocytes and metaphase II eggs were obtained as previously described (Coonrod et al., 1999, 2001).

Pronuclear embryos were obtained from females after injection of 10 I.U. of PMSG followed 48 h later by 10 I.U. of hcG and mating with ICR male retired breeders. Eighteen to twenty-four hours after injection of hCG, pronuclear staged embryos were isolated from the oviduct in Whitten's media with Hepes (Specialty media; Phillipsburg, NJ) containing 0.05% hyaluronidase. Embryos were washed thoroughly in TE media and either removed for fixation or allowed to develop in vitro at 37°C and 5% CO<sub>2</sub>. Oocytes and embryos were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Following fixation, oocytes and embryos were washed five times in PBS + 1% BSA (PBS/BSA) and then permeabilized with 0.5% Triton X-100 in PBS. Oocytes and embryos were then washed five times in PBS/BSA and blocked for 30 min with PBS/BSA containing 10% goat serum. Oocytes and embryos were then incubated with 40 µg/ml ePAD IgG or 40 µg/ml guinea pig IgG for 1 h at room temperature. Oocytes and embryos were washed five times and incubated for 1 h at room temperature with goat anti-guinea pig FITC-labeled secondary antibody (Jackson ImmunoResearch). Oocytes/embryos were mounted on slides and visualized under a Zeiss Standard 18 ultraviolet microscope.

#### *Confocal microscopy*

Eggs and embryos used for confocal microscopy were prepared as above except that in vivo derived embryos were used. The morula and blastocyst staged embryos were obtained by flushing the oviducts of mated females either 4 or 5 days following hCG injection and processed as above. Microscopy images were obtained on a Zeiss Axiovert 100 micro systems LSM confocal microscope. Each image was acquired using 4 s scan averaged four times per line using a 40× oil lens with a zoom of approximately 2. False color was added as appropriate.

#### *Electron microscopy*

Mouse ovaries were fixed for 2 h at room temperature in 8% paraformaldehyde buffered with 0.1 M sodium phosphate, pH 7.5. The ovaries were processed through a series of ethanol washes to dehydration and embedded with Lowicryl resin (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin sections were picked up on 75 mesh nickel grids and immunolabeled with primary antibody overnight at 4°C, washed, and secondary antibody on 5 nm colloidal gold was added to sections at room temperature for 45 min, washed, and heavy metal-stained with aqueous saturated Uranyl acetate for 30 min. The sections were viewed and photographed by using a JEOL 100CX2 electron microscope.

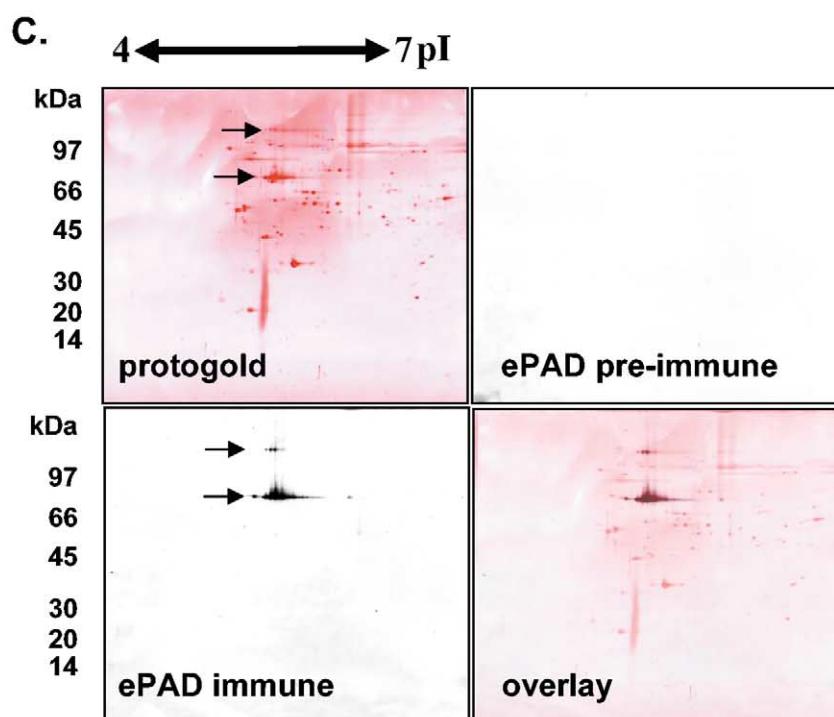
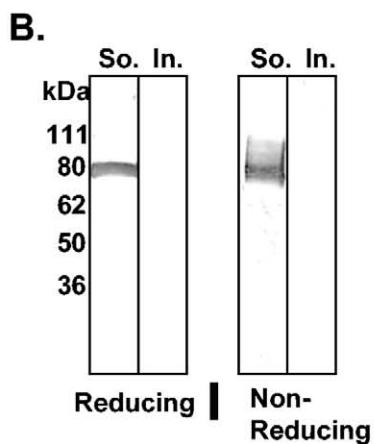
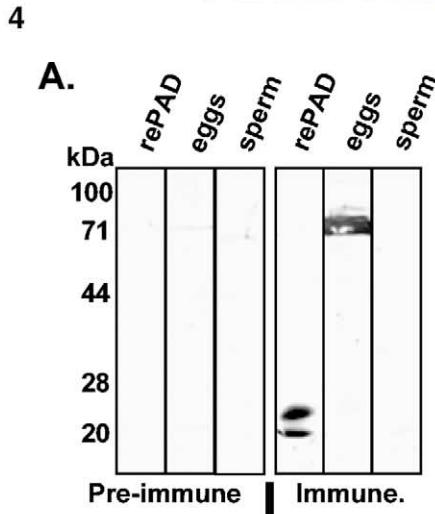
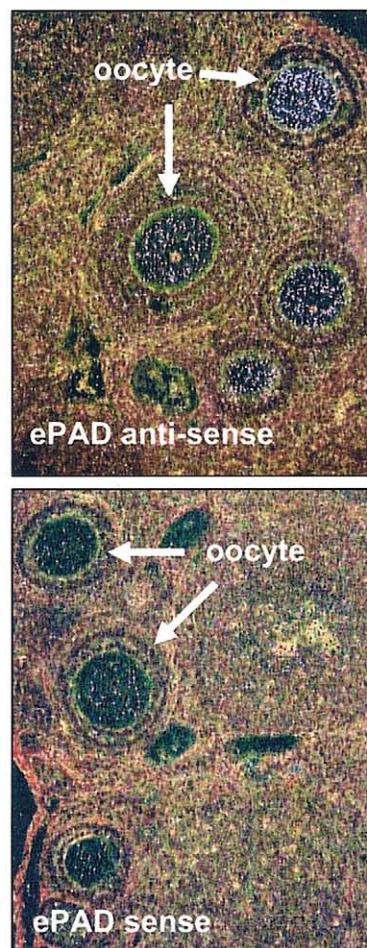
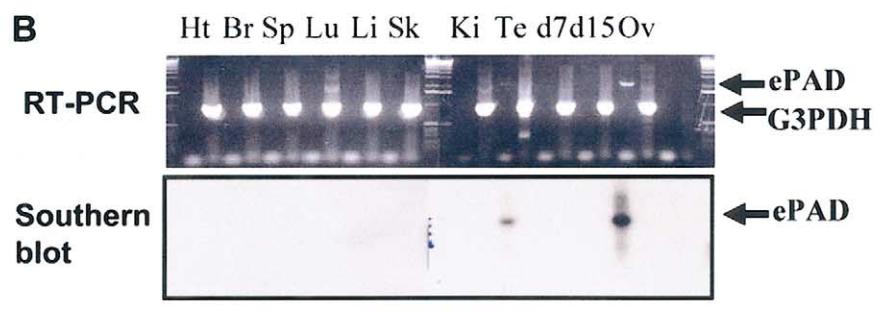
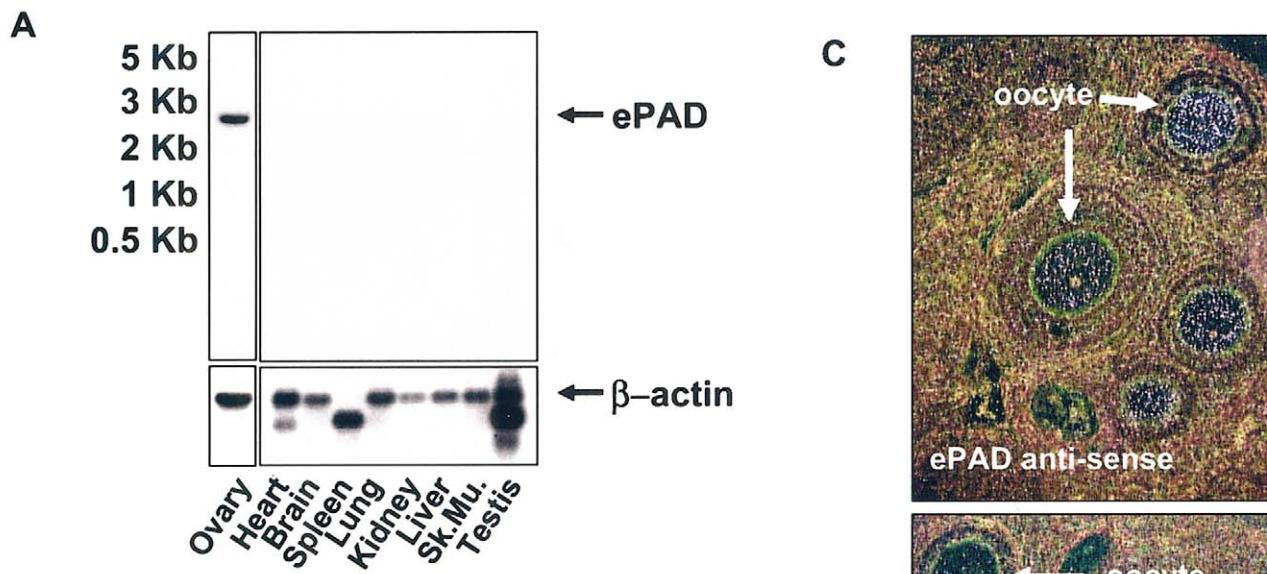
## Results

### *Tandem mass spectroscopic identification of ePAD, a highly abundant novel egg protein*

Zona-free mouse eggs (2850) were extracted, separated on a 2D electrophoretic gel, and stained with Coomassie (Fig. 1). Within a train of proteins (*pI* ~ 5 to 5.5) two spots, designated ePAD (Fig. 1, arrows) and migrating at ~75 kDa, were cored from the gel, digested with trypsin, and microsequenced by CAD Mass spectrometry. Twenty-eight peptides were identified in the spot indicated by the arrow on the left in Fig. 1, and 12 peptides were identified in the spot indicated by the arrow on the right, with 5 common peptides being shared between the 2 spots. The presence of common peptides within the 2 spots suggested that the entire protein train (Fig. 1, brackets) may arise from a single gene or related genes. Comparison of the staining intensity of this protein train with that of other protein spots found that ePAD appears to be one of the most abundant proteins in the ovulated mouse egg. When the identified peptides were searched against nonredundant database sequences, it was concluded that this protein had not been previously characterized. One of the common peptides having the sequence CCLEEKVCGLLEPLGLK, however, matched 2 EST clones arising from 2-cell (AA645498) and 8-cell (AU020751) mouse embryo cDNA libraries.

#### *Cloning and characterization of ePAD*

Repeated RACE PCR was performed by using an ovarian Marathon-ready adapter-primed cDNA library and oligonucleotide primers designed from the CCLEEKVCGLLEPLGLK peptide sequence, the matching EST clones, or gene-specific sequences resulting from amplification of 5' and 3' PCR gene fragments. Oligonucleotides were designed from the gene fragments and used to PCR-amplify a 2374-bp cDNA from the ovarian library. The gene was inserted into a cloning vector and subsequent DNA sequencing revealed a contiguous open reading frame of 2043 bp which encoded a 681-amino-acid protein (Fig. 2). The untranslated flanking regions are shown in lower case letters. The GenBank Accession No. for this clone is NP\_694746. The computed mass and isoelectric point of the deduced amino acid sequence is 76.7 and 5.36, respectively (ExPASy compute pi/MW tool), which closely matched the approximate mass and *pI* of the protein spots that were cored from the gel for TMS analysis. Nineteen of the 28 peptides originally identified by TMS were found in the deduced amino acid sequence and are underlined. When the deduced ePAD amino acid sequence was searched against the Prosite computational database, no highly significant structural motifs were observed. An NCBI conserved domain search using the deduced amino acid sequence, however, identified a peptidylarginine deiminase (PAD) domain (*E* value = 0.0) that spanned the protein sequence from the



seventh residue to the C terminus. Comparison of the sequence against the NCBI nonredundant database using the BLAST algorithm found that the sequence was 96% identical to a recently submitted putative mouse PAD type V-like protein sequence (XM\_144067), which was predicted by NCBI's automated sequence analysis program. The next most similar sequence was a predicted human putative human PAD type V-like protein sequence (XP\_017315.3) with an identity of 63%. ePAD was ~40% identical to all other characterized PAD sequences, including those of mice, humans, rats, sheep, and chickens. A direct comparison of ePAD with the four characterized mouse PADs using the ClustalW alignment function in the BioEdit sequence alignment editor indicated that ePAD is approximately 40% identical to the known PADs, while each of these PADs is approximately 50% identical to each other (Fig. 3). Interestingly, most of the domains that are conserved between the known PADs are also conserved in ePAD, indicating that ePAD likely represents a new member of the PAD enzyme family.

#### *ePAD is expressed in oocytes and early cleavage-stage embryos*

An EST database search using the ePAD open reading frame nucleotide sequence found nearly identical matches in mouse ovary, unfertilized egg, fertilized egg, 2-cell embryo, 8-cell embryo, and more recently, the day 0 neonatal thymus (data not shown). This information led to the hypothesis that ePAD may be an egg-abundant transcript whose expression persists throughout the early stages of cleavage. ePAD expression was examined in various tissues by using Northern blotting, RT-PCR, and *in situ* hybridization.

A multitissue Northern blot (Clontech) containing 5 µg poly(A)<sup>+</sup> RNA, and a separate blot containing 40 µg of total ovarian RNA, were probed with a <sup>32</sup>P-labeled random-primed 1100 bp ePAD N-terminal DNA fragment. A single 2.4-kb message was found to be abundantly expressed in the ovary (Fig. 4A). Subsequent multiple tissue blotting experiments using a single membrane showed similar results (data not shown). Ovary-abundant expression of ePAD was further confirmed by RT-PCR and Southern blotting (Fig. 4B). A mouse multiple tissue cDNA panel (Clontech) comprised of cDNA from 11 different tissues (including day 7 and day 15 embryos) was used as template in the PCRs. The oligonucleotide primers used for the PCRs encompassed the ePAD ORF (2043 bp). A strong ~2-kb amplicon was detected in the ovary cDNA lane, a weaker amplicon was seen in the testis cDNA lane, and no amplicons were detected in lanes containing cDNA from other tissues. To confirm the authenticity of the PCR amplicons seen in the ovary and testis lanes, the PCR products were blotted to a nitrocellulose membrane and the blot was probed with the same <sup>32</sup>P-labeled ePAD probe, which was used for Northern blotting. Results showed that the PCR products were derived from ePAD sequence (Fig. 4B.). A <sup>32</sup>P-labeled full-length β-actin probe and oligonucleotides designed to amplify the open reading frame of G3PDH were used as positive controls for the Northern blot and the RT-PCR, respectively.

*In situ* hybridization experiments were then performed to localize ePAD expression within the ovary. Results showed ePAD message only in oocytes (Fig. 4C) and not in other ovarian cell types. Further, ePAD message was not detected in oviductal or testis cross sections (data not shown). Taken together, these results suggest that ePAD is abundantly expressed in the ovary (specifically within oocytes) and may be expressed at lower levels in testis and thymus.

**Fig. 4.** Tissue distribution of ePAD. Northern blot analysis was performed on various tissues by using a P32-labeled 1100-bp ePAD DNA fragment (A). The Northern membrane contained 40 µg of total RNA for the ovary lane and 5 µg poly (A) RNA for the other tissues (exposure time 24 h). ePAD was detected as a single ~2.4-kb band in the ovarian RNA lane. A β-actin probe was used to control for RNA loading. RT-PCR and Southern blot analysis of ePAD tissue distribution (B). Specific primers based on the ePAD open reading frame were used in PCRs to screen cDNA from various tissues for ePAD expression. cDNA was tested from the following tissues: heart (Ht.), brain (Br), spleen (Sp), lung (Lu), Liver (Li), skeletal muscle (Sk), Kidney (Ki), testis (Te), day 7 embryo (d7), day 15 embryo (d15), and ovary (Ov). Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) primers were used as a positive control. A strong ePAD amplicon was seen in the ovarian RNA lane, while a weak amplicon was seen in the testis lane. A Southern blot was performed by using a <sup>32</sup>P-labeled ePAD DNA fragment to confirm the authenticity of the amplicon. *In situ* hybridization of mouse ovarian tissue with ePAD anti-sense RNA (C) demonstrated hybridizing to oocytes (white grains against dark field, indicated by arrows) but not to other ovarian cell types, while ePAD sense-strand RNA did not specifically hybridize to any ovarian tissues. Testis and oviductal sections were also tested with the ePAD probes and no signal was detected (data not shown). **Fig. 5.** A 75-kDa (pI 5.5) protein spot containing ePAD peptide sequence is specifically recognized by anti-ePAD antibodies. Antisera was generated in male guinea pigs against a purified recombinant form of ePAD containing the N-terminal 200 amino acids. The preimmune sera was not reactive with either the recombinant protein or with egg (50 mature zona-intact eggs per lane) or sperm proteins (~1 × 10<sup>6</sup> per lane), while the anti-ePAD sera was reactive with rePAD and with a ~75-kDa egg protein, but not with sperm protein (A). The solubility characteristics of ePAD were investigated, and the protein was found to partition with the 1% NP-10 soluble fraction (So.) and not with the insoluble fraction (In) under both reducing and nonreducing conditions (B). Three hundred ovulated zona-intact mouse oocytes were extracted, separated by 2D electrophoresis, blotted to nitrocellulose, and egg proteins were resolved with protogold. The blot was then probed with anti-ePAD preimmune IgG and prepared for protein detection by using enhanced chemiluminescence (ECL). The blot was then washed and reprobed with anti-ePAD immune IgG. Arrows indicate reactive proteins at ~75 and 110 kDa. Overlay image localizes reactive proteins with protein spots resolved using protogold (C). This Western blot demonstrates that the anti-ePAD IgG strongly reacts with the protein train that was originally core for microsequence analysis. In a separate experiment, ePAD peptides were also found by TMS at 110 kDa (data not shown), which may explain the observed upper reactive band (upper arrow).

*A 75-kDa (*pI* 5.5) protein spot which contains ePAD peptide sequence is specifically recognized by anti-ePAD antibodies generated against N-terminally truncated recombinant ePAD*

A cDNA encoding an N-terminal 200-amino-acid region of ePAD was cloned into a bacterial expression vector, expressed, purified, and used as an immunogen for anti-ePAD antisera production in guinea pigs. The reactivity of anti-ePAD antisera with the recombinant protein and with the appropriately sized egg protein was first confirmed by 1D Western blotting. The preimmune sera was not reactive with rePAD, egg, or sperm proteins. The immune sera was reactive with rePAD and an appropriately sized egg protein; however, the antibody did not react with sperm proteins (Fig. 5A). Previous experiments had shown that ePAD was soluble in several detergents (data not shown); this was confirmed by probing Western blots of egg proteins extracted 1% NP-40 and separated under both reducing and nonreducing conditions. As seen in Fig. 5B, ePAD is soluble under both conditions. It is of interest that a higher MW ePAD immunoreactive region appears under nonreducing conditions, possibly indicating a protein–protein interaction. Given that the anti-ePAD antisera appeared to be of high quality, the IgG fraction of the preimmune and immune sera was purified and used for all subsequent experiments.

On 2D immunoblots, the immune IgG to recombinant ePAD reacted with an ~75-kDa protein train at the precise location from which the two spots were originally cored, while the preimmune IgG was not reactive with any egg proteins (Fig. 5C). This finding provided an immunological proof that specific antibodies had been generated against the original cored protein, and that the cDNA and amino acid sequences obtained represented the intended spot. The train of ePAD isoelectric variants at 75 kDa is likely indicative of multiple posttranslational protein modifications. Lesser immunoreactivity was also noted with a protein train at ~125 kDa and *pI* 5.5 (Fig. 5C, upper arrow). In order to determine whether this immunoreactivity at 125 kDa represented authentic ePAD, bands in the 125-kDa MW range were microsequenced from a 1D gel, and ePAD peptide sequences

were noted, confirming that the band at 125 kDa in Fig. 5C represents a higher molecular weight form of ePAD (data not shown).

*Ontogenetic expression and subcellular localization of ePAD*

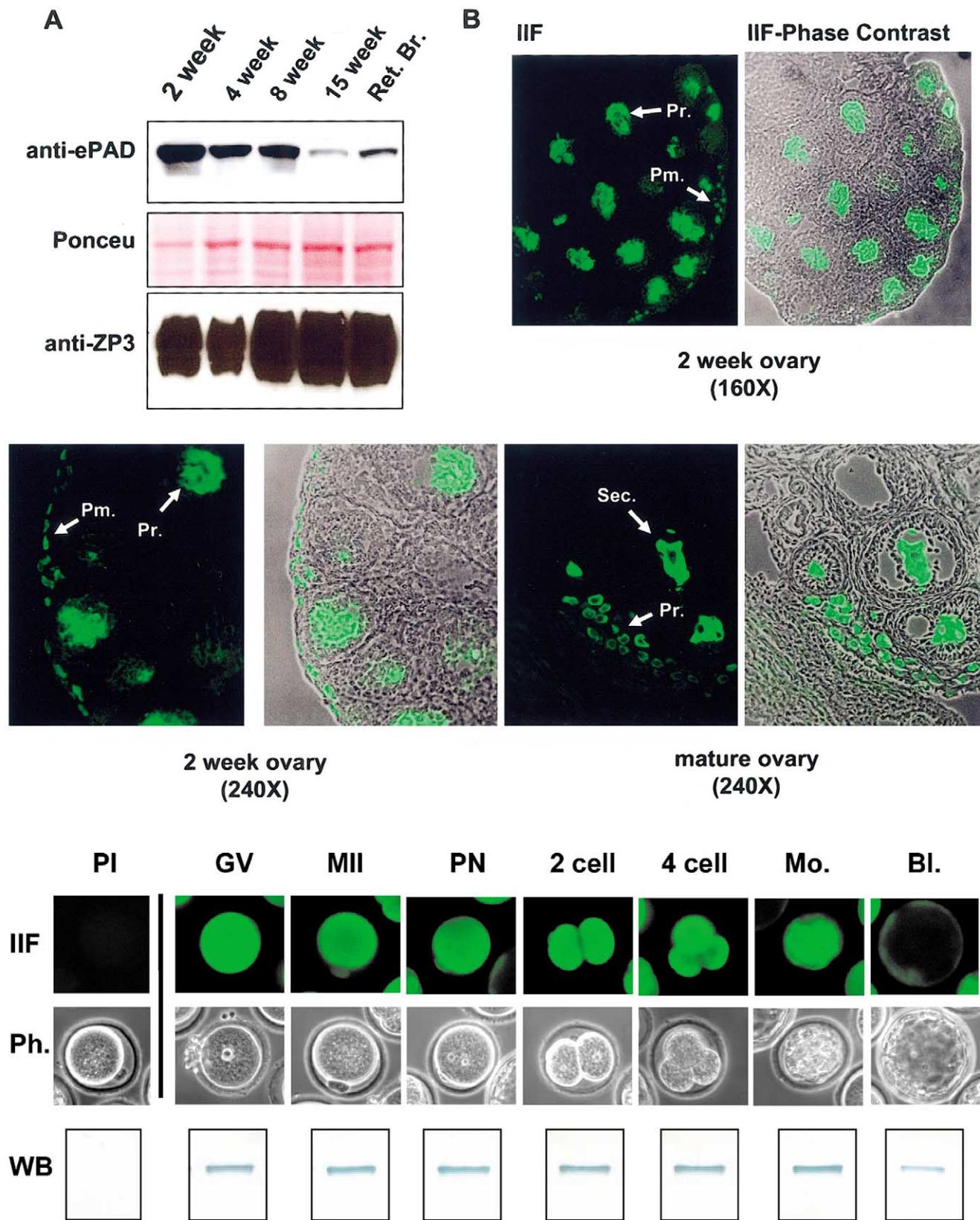
The monospecific IgG to rec ePAD was employed to investigate the developmental expression of ePAD. Ovaries were collected from 2, 4, 8, and 15 wk females, as well as from retired breeders and either embedded for sectioning and indirect immunofluorescence or proteins were extracted for Western blotting with equal concentrations of ovarian protein extract loaded in each lane. By Western blot (Fig. 6A), the ePAD protein was found at all stages examined. ePAD's relative abundance, however, appeared to be highest at 2 weeks of development (the earliest stage tested) and to gradually decrease as the mice aged. Since oocyte numbers are reduced over time through ovulation and atresia, and since ovarian structures such as corpora lutea and interstitial glands differentiate and regress, the ratio of oocytes to total ovarian tissue mass might be expected to vary at some stages. To control for this possibility, the blots were stripped and reprobed with antibodies to ZP3. Results showed that, as opposed to ePAD, the expression levels of this oocyte-specific protein appeared to remain constant or possibly increase as the ovaries developed and aged. Therefore, the observed decreases at 15 weeks and in retired breeders are unlikely to be the result of unequal numbers of oocytes represented in each sample.

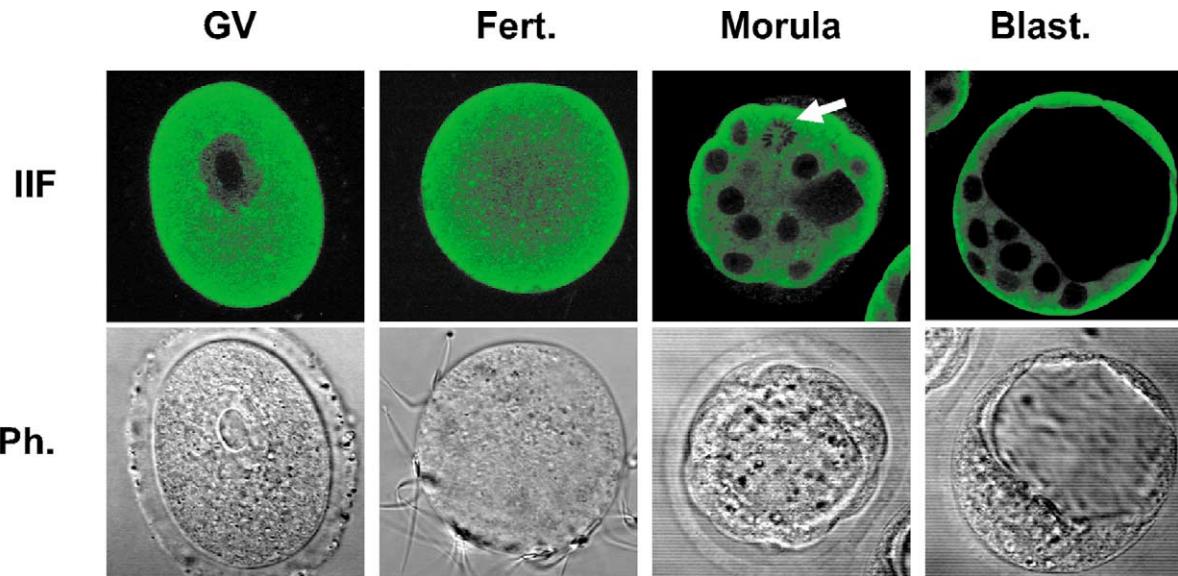
Indirect immunofluorescence of the ovarian cryostat sections confirmed that ePAD was expressed in an oocyte-specific manner in all types of follicles and in all developmental stages tested (Fig. 6B, stages other than 2 week and retired breeders are not shown). In 2-week-old females, abundant oocyte staining was observed in many primordial follicles (Pm.) and in all primary follicles (Pr.). When ovarian sections of mature females were stained with the anti-ePAD IgG, intense staining was observed in oocytes within primary, secondary (Sec.), and some primordial follicles. Staining was also observed in tertiary (Graafian) follicles

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Fig. 6. Ontogenetic expression of ePAD. Ovaries were collected from mice at different stages of development and either extracted for Western blot analysis (A) or prepared for indirect immunofluorescence (B). (A) Prior to Western blot analysis, blots were probed with Ponceau to control for protein loading. When blots of ovarian extracts were probed with anti-ePAD IgG, ePAD expression appeared highest in 2-week-old females and then declined slightly during animal growth. The blots were then stripped and reprobed with anti-ZP3 IgG in order to compare ePAD expression with that of another oocyte-specific protein. As opposed to ePAD, ZP3 expression appeared to increase slightly during animal growth. (B) Ovarian cross-sections from 2-week-old females and mature females were probed with anti-ePAD IgG and prepared for indirect immunofluorescence imaging (IIF) and phase contrast microscopy. ePAD expression in 2-week-old females was observed in eggs contained within primordial (Pm.) and primary follicles (Pr.). In mature ovaries, ePAD expression is seen in primary and secondary (Sec.) oocytes, while other ovarian tissues were not stained. Oocytes contained within tertiary follicles were also recognized by anti-ePAD IgG (Data not shown). These results further confirm the oocyte-abundant expression pattern of ePAD.

Fig. 7. Developmental expression of ePAD in the oocyte and early embryo. Indirect immunofluorescence (IIF) and Western blot (WB) analysis were performed to determine whether ePAD continues to be expressed in the developing embryo. Immature (GV), mature (MII), and pronuclear (PN) oocytes/embryos were collected from females, while 2-cell, 4-cell, morula (Mo.), and blastocyst (Bl.) were collected following culture from the pronuclear stage. Indirect immunofluorescence was performed on the eggs/embryos by using either ePAD preimmune IgG (PI, negative control, MII eggs) or immune IgG. Western blotting (WB) was also performed on each of the stages shown using 20 oocytes/embryos per group. ePAD appears to be expressed at all stages shown, with a decrease in expression levels being observed at the blastocyst stage.





**Fig. 8.** Scanning confocal analysis of ePAD subcellular localization. Immature oocytes (GV) were collected from ovaries. For fertilized eggs (Fert.), metaphase II eggs were collected from superovulated females, the zona pellucida were removed with chymotrypsin, and the eggs were coincubated with sperm for 45 min and fixed for evaluation. Morula and blastocysts were collected directly from females either 4 or 5 days after mating. In immature oocytes, ePAD appears to be predominantly expressed in the cytoplasm; however, some nuclear staining is also evident, while no staining is seen in the nucleolus. In fertilized eggs, ePAD is expressed in punctate patches throughout the cytoplasm. In the morula and blastocyst (Blast.), ePAD appears to be localized mainly to the cytoplasm; however, there does appear to be a limited amount of nuclear staining in some blastomeres. Note that the blastomere indicated by the arrow appears to be in metaphase and that ePAD is excluded from the chromatin-containing region. Also, cortical staining is seen at each of the developmental stages shown. In the morula, however, the cortical staining is limited to the external surface of the blastomeres and appears polarized. In the blastocyst, the cortical staining is mainly limited to the trophectoderm. IIF, indirect immunofluorescence; Ph., phase contrast.

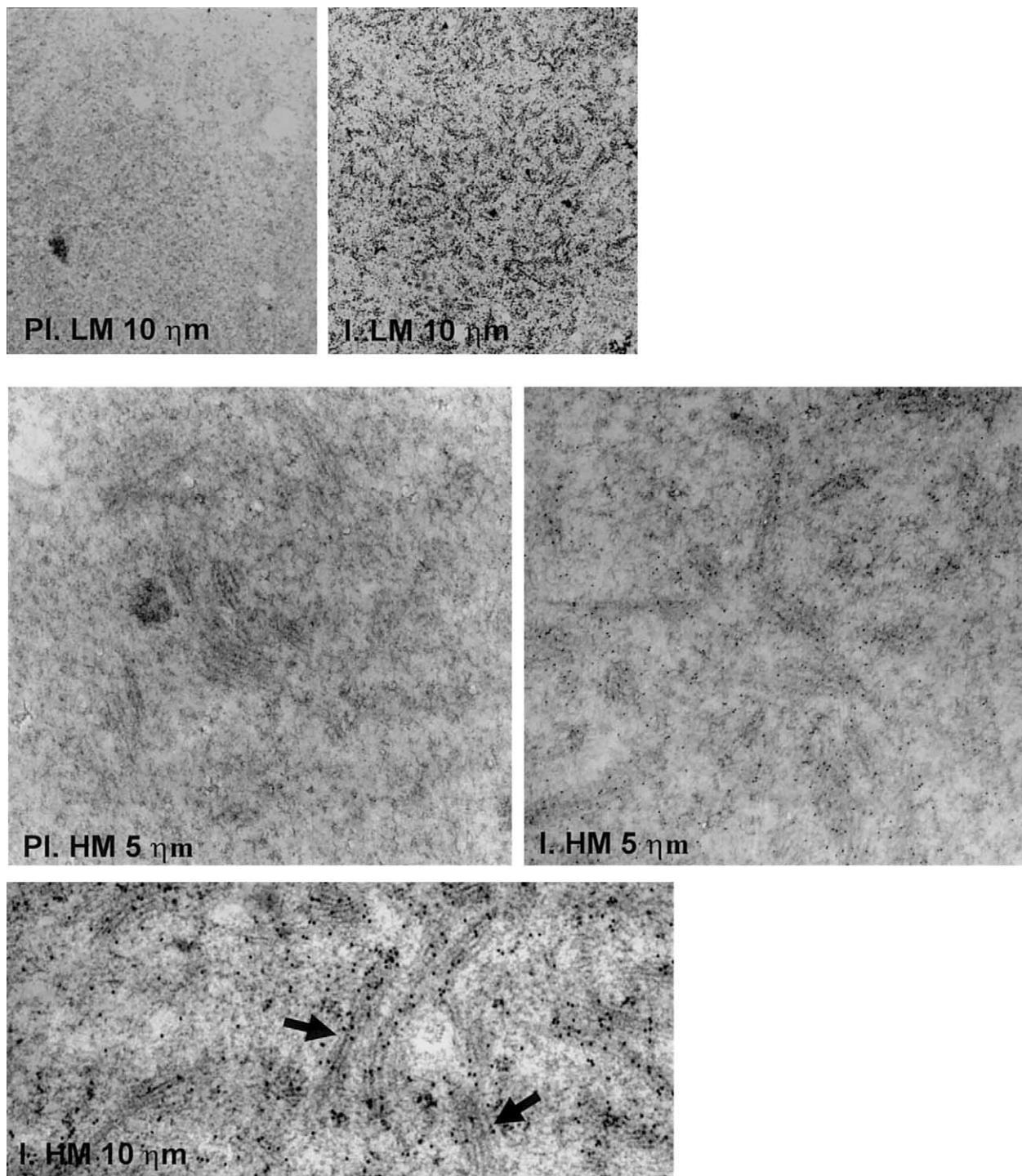
(data not shown). No ovarian cell types other than the oocytes were reactive with the antibody.

The expression of ePAD protein during oocyte maturation, fertilization, and early embryonic development was next investigated. Immature germinal vesicle-stage oocytes (GV), mature metaphase II arrested eggs (MII), and pronuclear zygotes (PN) were collected from females and immediately fixed. Pronuclear-stage embryos were also collected and cultured in vitro, and the cultured embryos were harvested at the 2-cell, 4-cell, morula (Mo.), and blastocyst (Bl.) stage and fixed for immunocytochemistry. All stages were then permeabilized, stained with secondary antibody alone, anti-ePAD preimmune IgG, or anti ePAD immune IgG, and analyzed by indirect immunofluorescence. No staining was observed when eggs were stained with anti-ePAD preimmune IgG (Fig. 7, PI). Further, neither the anti-ePAD preimmune IgG or the secondary antibody alone stained any of the other stages tested (data not shown). Anti-ePAD immune IgG immunofluorescence staining indicated that ePAD protein was present at all stages, with a more heterogeneous staining pattern seen among the blastomeres at the morula stage and an apparent decrease in staining intensity seen at the blastocyst stage (Fig. 7). To test whether the ePAD protein detected by microscopic methods could be confirmed biochemically, 20 eggs/embryos were collected for each stage as described, extracted, and blotted to a nitrocellulose membrane. The blot, probed with anti-ePAD antibody, confirmed that ePAD was present

until the blastocyst stage, at which point ePAD expression decreased (Fig. 7, WB).

Confocal analysis was then performed to further investigate the subcellular localization of ePAD (Fig. 8). Evaluation of immature oocyte (GV) cross sections showed that ePAD localized predominantly to the cortical region of the oocyte, with decreased staining in interior cytoplasmic regions. Low levels of ePAD staining were noted in the oocyte nucleus, with complete exclusion of staining from the nucleolus. Close examination of the oocyte cytoplasm revealed that ePAD staining was punctate in nature. This pattern of punctate staining suggested that ePAD might be associated with a specific cytoplasmic element. In fertilized eggs (Fert.), ePAD was expressed in punctate patches throughout the cytoplasm. In the morula and blastocyst (Blast.), ePAD appeared to localize mainly to the cytoplasm; however, a limited amount of nuclear staining was noted in some blastomeres. Note that the blastomere indicated by the arrow (Fig. 8) appeared to be in metaphase and that ePAD was excluded from chromatin-containing regions. Intense cortical immunofluorescent staining was seen at each of the developmental stages shown, except in the morula, in which cortical staining was limited to the external surface of the blastomeres and appeared polarized. In the blastocyst, the cortical staining was mainly limited to the cells of the trophectoderm (Fig. 8).

To investigate ePAD localization at the ultrastructural level, immunoelectron microscopy was performed by using



**Fig. 9.** Immunoelectron microscopic localization of ePAD in oocytes. At low magnification (LM) using 10-nm-labeled gold particles, few preimmune-coated (PI) particles are seen in a cytoplasmic section of an egg, while immune-coated particles (I) are found to be abundantly associated with the egg cytoplasm in a swirling pattern. Higher magnification (HM) using 5-nm gold particles reveals few preimmune particles in the cytoplasmic region of the egg, while numerous immune particles predominantly localize to the cytoplasmic sheets of the egg. At high magnification, 10-nm particles coated with anti-ePAD IgG clearly localize to the sheets. This cytoskeletal structure is unique to the mammalian egg and embryo and is thought to be mainly composed of intermediate filaments.

anti-ePAD-coated 10-nm gold particles to probe ovarian and cumulus–oocyte complex cross-sections (Fig. 9). Comparison of low magnification fields revealed that the preimmune IgG-coated gold particles did not bind to the egg

cytoplasm, while the anti-ePAD IgG gold particles localized within the egg cytoplasm to whorls of electron dense material. Anti-ePAD immunogold particles were not found to specifically associate with any other ovarian cell type in-

vestigated, nor did the particles appear to specifically associate with electron lucent regions of cytoplasm. At higher magnifications, anti-ePAD immunogold particles localized to multilamellar filamentous cytoskeletal structures that have been previously named cytoplasmic sheets. These cytoskeletal specializations are distinctive in their lamellar appearance within the cytoplasm of the egg and early embryo (Capco et al., 1993).

## Discussion

Our laboratory has been utilizing proteomics to characterize proteins in the mouse egg in an effort to identify new potential contraceptive targets. We chose to clone and characterize ePAD based on the observation that it represented one of the most abundant proteins yet to be characterized in the mouse egg. The specific localization of ePAD to oocytes in ovarian sections and its homology to a well-characterized enzyme family that has known in vitro and in vivo substrates supports further development of small molecule inhibitors to this contraceptive target.

A central question germane to the potential role of ePAD in egg physiology is whether ePAD functions as an egg and/or embryo peptidylarginine deiminase, and if so, what are its substrates? Previous experiments have shown that the cytoplasmic sheets consist of a highly cross-linked network of Triton X-100 detergent-insoluble intermediate filaments that are coated with a Tween 20 detergent-soluble protein component. The most abundant soluble component of the sheets is an ~69-kDa protein that has not been characterized at the molecular level (Gallicano et al., 1994a). Given ePADs localization to the cytoskeletal sheets, its solubility characteristics, and its molecular weight (75 vs 69 kDa), it is tempting to speculate that ePAD is the soluble ~69-kDa protein previously described in the literature (Gallicano et al., 1994a). The molecular nature of the insoluble component has been partially characterized and shown to contain cytokeratin (Gallicano et al., 1994a). Because keratin is such a well-characterized PAD substrate in epithelial cells (Senshu et al., 1999a,b), it represents an obvious potential substrate for ePAD in eggs and embryos.

In epithelial cells, deimination of specific arginine residues in keratin and filaggrin is known to be involved in the conversion of a preexisting fine cytokeratin network to more densely bundled linear filamentous structures (Ishida-Yamamoto et al., 2002). This reorganization is reminiscent of changes that have been described in the cytoskeletal sheets in the egg where the preexisting multilamellar, whorl-like pattern in unfertilized eggs (similar to that seen in Fig. 9) becomes more linearly arranged in the fertilized zygote (Gallicano et al., 1995). At compaction, the sheets appear as highly polarized structures with one end anchored on the apical plasma membrane and extending downward toward the basolateral surface within each blastomere. As with ePAD protein expression, by the blastocyst stage of

development, the sheet density begins to diminish. In the trophectoderm, the sheets are localized near the plasma lamina, while in the inner cell mass blastomeres, the sheets tend to be more centrally located (Capco et al., 1993).

Because PADs are calcium-dependent enzymes, fertilization represents a particularly interesting developmental stage for potential ePAD activity since large calcium transients are known to occur following sperm–egg fusion (Carroll, 2001). Following fertilization, one could imagine that the calcium transients could activate ePAD, which would then deiminate keratin, resulting in the observed cytoskeletal sheet reorganization. Such reorganization may be essential for sperm internalization or polar body extrusion.

In addition to the keratins of the cytoskeletal sheets, the nuclear localization of ePAD suggests that other proteins also represent potential ePAD deimination targets at fertilization. For example, protamine, an arginine-rich (~60% arginine residues) sperm-specific histone-like protein, is known to be an excellent in vitro substrate for PADs (Sugawara et al., 1982). Given that substrate deimination causes changes in secondary structure (Tarcza et al., 1996), deimination of sperm protamine by ePAD, following gamete fusion, might facilitate nuclear decondensation. In fact, we have found that commercially available skeletal muscle PAD readily decondenses sperm chromatin in vitro (data not shown). Further experiments are being performed to determine whether either native or full-length recombinant ePAD can decondense sperm in vitro and in vivo.

Histone proteins in HL-60 granulocytes have recently been found to contain citrulline residues and therefore are likely substrates for PADs (Hagiwara et al., 2002). The investigators who made this observation hypothesized that the N-terminal tails represent the most likely site for PAD activity, and if this prediction is correct, deimination of the histone tails represents a novel posttranslational histone modification. Other N-terminal histone modifications, such as acetylation, phosphorylation, and methylation, have been shown to alter chromatin structure and thus lead to changes in transcriptional activity of specific genes (Jenuwein and Allis, 2001). It is well documented that there is a dramatic reprogramming of gene expression patterns following fertilization and prior to zygotic gene activation; however, the mechanism by which the egg is able to perform this remarkable task is unknown. The egg factors involved in this process are likely to be cytoplasmic, as demonstrated by the observation that somatic cell reprogramming requires that the nuclei be placed in the context of the egg cytoplasm (Surani, 2001). It will be of interest to establish whether ePAD can deiminate egg/embryo histones and thus possibly play a role in the reprogramming process.

To conclude, we have cloned and characterized ePAD, a highly abundant egg and embryo protein that is ~40% identical to the peptidylarginine deiminase enzyme family. ePAD appears to be expressed from the primary oocyte stage of oogenesis until at least the blastocyst stage of development. At the ultrastructural level, ePAD localizes to

the egg cytoplasmic sheets, keratin-containing structures known to undergo changes in their structure during early development. As one of the most abundant proteins in the egg proteome, further consideration of the functions of this potentially novel deiminase are germane to fertilization and early development.

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