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Mouse sperm-egg plasma membrane interactions: analysis of roles of egg integrins and the mouse sperm homologue of PH-30 (fertilin) β

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SUMMARY

The guinea pig sperm protein, PH-30 (also known as fertilin), is postulated to participate in the interaction between the sperm and egg plasma membranes. The β subunit of guinea pig PH-30 (gpPH-30β) contains a domain with homology to disintegrins, snake venom proteins that bind to integrins via an integrin-binding domain containing the tripeptide RGD. This raises the question of whether an egg integrin serves as a receptor for PH-30. Although mouse eggs express integrin subunits, their role in mouse fertilization is unresolved. Therefore, we examined fertilization for two different hallmarks of integrin function, namely, dependence of ligand binding on divalent cations and the ability to inhibit ligand binding with RGD peptides. We demonstrate that sperm binding to *zona pellucida*-free eggs is supported by Ca^{2+}, Mg^{2+}, or Mn^{2+}. Ca^{2+} was necessary and sufficient for sperm-egg fusion, with 2.5 mM Ca^{2+} being the most effective concentration. In addition, fertilization could be partially inhibited with various RGD peptides, which caused a decrease in sperm-egg fusion by 30-58%. This partial inhibition of fusion with RGD peptides prompted the cloning of the mouse homologue of gpPH-30β (hereafter referred to as mPH-30β) to determine if it possessed the tripeptide RGD or a different amino acid sequence in its disintegrin domain. mPH-30β, which is expressed during meiotic and post-meiotic phases of spermatogenesis, shares significant similarities to gpPH-30β throughout the length of the molecule, from the signal sequence to the cytoplasmic tail. The full-length deduced amino acid sequence of mPH-30β is 55% identical and 72% homologous to gpPH-30β. The disintegrin domain of mPH-30β has the tripeptide QDE (instead of RGD) in its cell recognition region. Peptides containing this QDE sequence decrease the binding and fusion of sperm with *zona pellucida*-free eggs by approximately 70%, suggesting that the disintegrin domain of mPH-30β participates in the interaction between sperm and egg membranes.

Key words: RGD peptide, divalent cation, integrin, disintegrin, mouse egg, fertilization, calcium, fertilin, PH-30

INTRODUCTION

Sperm interact with mammalian eggs at two discrete and spatially separated sites. First, the acrosome-intact sperm binds to the *zona pellucida* (ZP), the extracellular coat that surrounds the egg and is composed of three glycoproteins called ZP1, ZP2, and ZP3. Binding of acrosome-intact sperm and induction of the acrosome reaction of those bound sperm is mediated by ZP3 (Wassarman, 1990). Although the sperm receptor for ZP3 is not known, several candidates have been proposed (Leyton and Saling, 1989; Bleil and Wassarman, 1990; Miller et al., 1992). Following passage through the ZP, the acrosome-reacted sperm binds to and fuses with the egg membrane, thus introducing the sperm nucleus into the egg cytoplasm. In contrast to what is known about ZP3-sperm binding, information regarding the molecular basis of membrane interactions between sperm and egg is much less complete. Sperm binding to and fusion with the egg occurs at discrete regions on both the sperm and egg plasma membranes. The equatorial segment of the sperm head, which constitutes the post-acrosomal region toward the posterior of the sperm head, appears to be the region of the sperm membrane that is involved in binding and fusion with the microvillar region of the egg plasma membrane (the region around the entire circumference of the egg with the exception of the area overlying the spindle) (Yanagimachi, 1994). To date, however, no specific molecules on mouse eggs or sperm have been clearly shown to participate in gamete membrane interactions.

Work in a different system, the guinea pig, has identified a sperm protein known as PH-30 or fertilin, which has properties expected of a candidate for mediating both gamete membrane binding and fusion. Guinea pig PH-30 (gpPH-30) is localized to the post-acrosomal region in acrosome-reacted sperm, and anti-gpPH-30 monoclonal antibodies inhibit the fusion of guinea pig sperm with eggs, thus blocking fertilization (Primakoff et al., 1987). gpPH-30 is a heterodimer composed of two integral membrane proteins. The α subunit contains a putative viral fusion peptide domain, suggesting a role for this subunit in sperm-egg membrane fusion, and the β subunit contains a disintegrin domain (Blobel et al., 1992).

Disintegrins, a family of soluble peptides from snake venoms, contain an amino acid sequence that resembles the Arg-Gly-Asp (RGD)-containing sequence present in several extracellular matrix (ECM) ligands for integrins. These molecules can antagonize specific integrin-ECM interactions (Gould et al., 1990). PH-30, therefore, could conceivably function as a sperm ligand that mediates both sperm-egg membrane binding and fusion, although only a role in fusion has been demonstrated (Primakoff et al., 1987; Myles et al., 1994). The presence of a disintegrin domain in gpPH-30 suggests that an egg integrin might serve as the receptor for sperm.

Integrins are heterodimers of noncovalently linked α and β subunits that function as cell adhesion molecules, and serve as receptors for a variety of ligands including extracellular matrix proteins and other cell adhesion molecules (Hynes, 1992; Haas and Plow, 1994). Moreover, integrins can participate in transduction of signals initiated by ligand binding (Juliano and Haskill, 1993). There are multiple hallmarks of integrin function. One of these is divalent cation dependence. Many integrins require divalent cations for ligand binding, and several integrins have distinct patterns of ligand binding activity in the presence of one divalent cation versus another. A second characteristic of integrin function is RGD dependence, since RGD-containing peptides can disrupt the interaction of several integrins with their ligands. In fact, RGD peptides inhibit binding and fusion of human and hamster sperm with *zona*-free hamster eggs (Bronson and Fusi, 1990).

We and others have shown that mammalian eggs express a variety of integrins (Anderson et al., 1993; Evans et al., 1995; Fusi et al., 1993; Hierck et al., 1993; Sutherland et al., 1993; Tarone et al., 1993). To extend our previous studies, we examined mouse fertilization for these hallmarks of integrin function. We demonstrate that sperm binding to ZP-free eggs occurs in Mg^{2+}, Mn^{2+}, or Ca^{2+}. In contrast, Ca^{2+} in the absence of Mg^{2+} and Mn^{2+} supports both binding and fusion of sperm and egg plasma membranes; fusion is maximal in 2.5 mM Ca^{2+}. Moreover, sperm-egg fusion is moderately inhibited (30-58%) by RGD peptides.

In an attempt to determine why RGD peptides were only moderately effective in inhibiting sperm-egg fusion, we cloned and sequenced the mouse homologue of gpPH-30β (mPH-30β). mPH-30β is highly homologous (72% at the amino acid level) to gpPH-30β, and is expressed in testis but not other tissues (brain, spleen, liver), and in male germ cells during meiotic and post-meiotic stages of spermatogenesis. cDNA sequence analysis also reveals that the disintegrin domain of mPH-30β contains the tripeptide QDE, instead of RGD. QDE peptides reduce sperm binding and consequently reduce the incidence of fertilization by 65-70%.

MATERIALS AND METHODS

Peptides

RGD peptides (GRGDTP, GPenGRGDSPCA (hereafter referred to as cRGD; Pen = penicilliamine), GRGDSP, and the control peptide, GRDESP) were purchased from Gibco-BRL (Gaithersburg, MD). All were purified by HPLC to >98% purity. The peptides were resuspended in 10 mM HEPES, pH 7.4, at a concentration of 50 mM or 100 mM, and stored in 10 μl aliquots at –20°C.

mPH-30β peptides were synthesized by Quality Controlled Biochemicals (Hopkinton, MA) and purified by HPLC to >98% purity. CAQDEC and CDEQAC were used in both linear and cyclized forms. To prepare linear peptides, CAQDEC and CDEQAC were resuspended in degassed deionized water at 5 mM, immediately distributed into 10-20 μl aliquots, and then dried down under reduced pressure. Dehydrated peptides were resuspended at a concentration of 50 mM in degassed 10 mM HEPES, pH 7.4, immediately before use. This preparation resulted in 80-90% of the peptide remaining unoxidized (linear) as determined by Ellman reaction (Adam et al., 1992). To prepare cyclized peptides, CAQDEC and CDEQAC were oxidized by an overnight incubation with vigorous shaking at a concentration of 1 mg/ml in either water or 25 mM NH_4OH. Oxidation in water or ammonium hydroxide resulted in 0-30% or 70-80% oxidation, respectively. Peptides covalently cyclized through a terminal bromo-acetyl group were also synthesized by Quality Controlled Biochemicals (BrAcAQDEC, hereafter referred to as cQDE, and BrAcQEADC, hereafter referred to as cQEAD).

Gamete preparation

Female six-week-old CF1 mice were primed with 5 i.u. of pregnant mare's serum gonadotropin (PMSG; Sigma, St Louis, MO) and then induced to ovulate with 5 i.u. of human chorionic gonadotropin (hCG, Sigma) 46-48 hours later. Cumulus enclosed-egg complexes were collected from the oviducts 12-13 hours after hCG administration, and treated with 0.05% hyaluronidase (Sigma) in Waymouth's medium (Gibco-BRL) containing 10% fetal calf serum to disperse the cumulus cells. The ZP was removed by a brief incubation in low pH buffer (acidic MEMCO, pH 1.5; Evans et al., 1995), and the eggs were then allowed to recover for 30-60 minutes by incubating them in Whitten's medium (Whitten, 1971) in an atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 37°C.

Sperm were collected from (C57BL/6J × SJL/J)F_1 male mice (8-10 weeks old) by placing the cauda epididymides and vasa deferentia in 900 μl of Whitten's medium supplemented with fatty-acid poor BSA at 15 mg/ml (ICN, Costa Mesa, CA) under mineral oil; sperm were allowed to swim out for 10-15 minutes, and then the tissue was removed from the medium. Sperm were capacitated for 2-3 hours; the percentage of spontaneously acrosome-reacted sperm was very constant (20%) due to the consistency of our capacitation conditions (Ward and Storey, 1984).

In vitro fertilization and assessment of sperm binding and fusion

The methods for in vitro fertilization (IVF) described by Moore et al. (1993) were used with the following modifications. Initial experiments were conducted to determine the relationship between sperm concentration and the efficiency of fertilization of ZP-free eggs in vitro (Table 1). For studies of the divalent cation dependence of fertilization, 2× concentrated divalent cation-deficient Whitten's medium was prepared. Ca^{2+}, Mg^{2+}, or Mn^{2+} was added (from 1 M stock solutions) to the concentrations indicated in the figures, and then the final concentration of Whitten's medium was adjusted to 1× with water. Sperm were diluted to 10,000-20,000 sperm/ml in these media after the above-described incubation period in Whitten's medium to allow sperm to capacitate and undergo the spontaneous acrosome reaction. Fertilization drops were prepared from these sperm suspensions (10,000-20,000/ml) as 8-10 μl drops, covered with mineral oil, and incubated at 37°C in an atmosphere of 5% CO_2, 5% O_2, 90% N_2. Prior to addition to these fertilization drops, the eggs were washed through three 100-200 μl drops of the appropriate medium. Eggs and sperm were co-incubated in 8-10 μl drops (one egg per μl of fertilization drop) for 1.5 hours. Control fertilizations were routinely performed in standard Whitten's medium with sperm concentrations of 50,000-100,000/ml to ascertain egg quality and to verify that >80% of the eggs could be fertilized.

For studies of peptide inhibition, methods similar to those of

Table 1. Parameters for in vitro fertilization of *zona*-free mouse eggs

Sperm concentration	Percentage of eggs fertilized	Average number of sperm bound per egg	Average number of sperm fused per egg
5,000-9,000/ml	42.88±4.65	0.89±0.10	0.44±0.05
10,000-12,500/ml	51.33±4.50	1.31±0.14	0.56±0.06
14,000-19,500/ml	61.06±6.22	2.08±0.41	0.73±0.09
20,000-25,000/ml	77.20±5.26	2.81±0.42	0.99±0.09
50,000-200,000/ml	83.00±6.40	8.09±0.76	1.07±0.11

Effect of sperm concentration on sperm-egg binding and fusion resulting from in vitro fertilization of *zona pellucida*-free mouse eggs. ZP-free mouse eggs were inseminated in Whitten's medium using the indicated concentrations of sperm (given in sperm/ml). The results here represent the average ± s.e.m. of 10-20 experiments per range of sperm concentrations and a total of 150-350 eggs fertilized per range of sperm concentration. It should be noted that these experiments were conducted with eggs from CF-1 mice and sperm from (C57BL/6J × SJL/J)F_1 mice and the results may vary with gametes from different strains.

Bronson and Fusi (1990) and Myles et al. (1994) were used. ZP-free eggs were initially incubated in different concentrations of peptide (0.05-2.5 mM as indicated in the figures) in Whitten's medium containing 15 mg/ml BSA for 60 minutes prior to co-incubation with capacitated sperm. Sperm were diluted to 5,000-20,000 sperm/ml in Whitten's medium containing BSA and the appropriate concentration of peptide. Eggs and sperm were co-incubated in 8-10 μl drops (one egg per μl of fertilization drop) for 1.5 hours. Control fertilizations were routinely performed with sperm concentrations of 50,000-100,000/ml to ascertain egg quality and to verify that >80% of the eggs could be fertilized.

After incubation with sperm, eggs were washed three times in Whitten's medium containing 15 mg/ml BSA to remove any loosely attached sperm; all washes were done by the same person using the same pipet and the same washing pressure. Eggs were then fixed in freshly prepared 3.7% paraformaldehyde in PBS for 15-30 minutes. Eggs were washed twice in PBS, and then permeabilized in PBS containing 0.1% Triton X-100 for 3-5 minutes. Following one wash in PBS and one wash in PBS containing 0.1% BSA, 0.01% Tween-20 (PBS-BSA-Tween), the eggs were then stained with DAPI (4′,6-diamidino-2-phenylindole; Sigma; 2 μg/ml in PBS-BSA-Tween) for 15-30 minutes, washed 3-4 times in PBS-BSA-Tween, and mounted in Vecta-Shield (Vector Laboratories, Burlingame, CA). Eggs were viewed by epifluorescence for the presence of decondensing sperm DNA within the egg cytoplasm. The percentage of eggs fertilized via fusion with one or more sperm (fertilization rate; Myles, 1993) and the average number of sperm fused per egg (fertilization index; Myles, 1993) were used to assess sperm-egg fusion. The number of sperm bound to the egg surfaces were also counted (in the data presented, this value includes the sperm that have already fused, based upon the assumption that these sperm were bound to the surface prior to fusion).

Assessment of fertilization was also performed by loading eggs with Hoechst 33342 (1 μg/ml) prior to co-incubation with sperm and observing the transfer of the DNA-specific dye to the sperm nucleus as it is incorporated into the egg cytoplasm (Hinkley et al., 1986). We found that identical results were obtained with the DAPI-staining method described above and that the DAPI-staining method was preferable for the processing of a large number of samples. In selected experiments, fixed eggs were also stained with the monoclonal antibody HS-19 that stains the outer acrosomal membrane of sperm (Florman et al., 1984). This procedure allowed identification of any non-acrosome-reacted sperm that were non-specifically sticking to the egg surface, since acrosome reacted sperm do not stain with this antibody. We consistently observed that 95-100% of the bound sperm were acrosome reacted.

Polymerase chain reaction

Degenerate primers were designed based on the guinea pig PH-30β nucleotide sequence (Blobel et al., 1992; Wolfsberg et al., 1993). The 5′ primer was TG[C/T]TG[C/T]GA[C/T]GC[C/T]GC[C/T]ACCTG, corresponding to nucleotides (nt) # 1233-1252 in the guinea pig sequence. The 3′ primer was CA[A/G/T]ATNAGTTTNCCACACTT, where N = all four nucleotides; this primer corresponds to nt # 1620-1639 in the guinea pig sequence. These primers were used to amplify a 406 bp fragment from a guinea pig testis cDNA λgt11 library (gift of George Gerton, University of Pennsylvania). λ phage were diluted 1:400 in 1× PCR buffer and phage particles were disrupted by incubating at 70°C for 5 minutes prior to PCR. Standard PCRs were run as 100 μl reactions as described previously (Temeles et al., 1994). A Perkin-Elmer-Cetus GeneAmp PCR system 9600 thermocycler (Norwalk, CT) was used to run the following program: one cycle of 94°C for 60 seconds; sixty cycles of 42°C for 60 seconds, 72°C for 30 seconds, and 95°C for 30 seconds; and a final extension cycle of 72°C for 5 minutes. The 406 bp fragment amplified in this reaction was re-amplified, and then cloned into pCRII using the TA Cloning kit according to manufacturer's instructions (Invitrogen, San Diego, CA). Plasmid was purified from bacterial cultures using a commercial plasmid preparation kit (Five Prime-Three Prime, Boulder, CO), and the presence of an insert was verified by *Eco*RI digestion. The identity of this insert was verified by sequencing using the M13 forward and reverse primers on an ABI automated DNA sequencer.

cDNA library screening and 5′ end extension

Gel-purified 406 bp plasmid insert, which corresponded to the PCR-amplified fragment of the guinea pig PH-30β (described above), was used as template for radiolabelling by priming with random hexamers, Klenow DNA polymerase, and [α-^{32}P]dCTP (US Biochemicals). Radiolabelled DNA was separated from unincorporated nucleotides on a G-50 Sephadex column (Five Prime-Three Prime, Boulder, CO). One hundred thousand pfu of a mouse spermatogenic cell λzap library (Carrera et al., 1994) were screened with this probe under moderately stringent conditions (5× SSPE (1 M NaCl), 55°C, according to the methods of Sambrook et al. (1989)). Positive plaques were verified by a secondary screen. Single positive plaques were then selected, and the phagemids isolated by in vivo excision according to manufacturer's instructions (Stratagene, San Diego, CA). Phagemids were isolated from bacterial cultures using a commercial plasmid preparation protocol (Five Prime-Three Prime), and the inserts were released from isolated phagemids by double digestions with *Sac*I and *Kpn*I. DNA sequencing was performed on an ABI automated sequencer. Initial sequence data were obtained from two different phagemids as described above with primers corresponding to the flanking T3 and T7 polymerase promoters. Subsequent sequence data from plus and minus strands of these two phagemids were obtained by sequencing with oligonucleotides designed to match the phagemid insert sequence. (Hereafter, the nucleotide sequence for mPH-30β is numbered counting the first nucleotide of the start codon as '1.')

DNA sequence analysis of the longest cDNA isolated via the initial screen was not full length. To extend the 5′ sequence, the '5′ inverse PCR' method of Zeiner and Gehring (1994) was used with the following modifications. First strand cDNA was synthesized from mouse testis total RNA with a gene-specific primer (CCGGTCTG-GCTTGATACTTC, corresponding to nt #476-495 on the mPH-30β sequence) using Superscript II (Promega) reverse transcriptase (Temeles et al., 1994). Second strand cDNA was synthesized at 14°C for 16 hours in a 100 μl reaction using 23 units *E. coli* DNA polymerase I (New England Biolabs) in 1× New England Biolabs polymerase buffer supplemented with 150 μM NAD^+ (Boehringer Mannheim), 2 units *E. coli* DNA ligase (New England Biolabs), and 1 unit of RNAse H (Promega). DNA was phenol-extracted and

ethanol-precipitated, and then blunt ends were generated with 2 units of Klenow DNA polymerase in 1× New England Biolabs polymerase buffer supplemented with 0.25 mM dNTPs. This double-stranded cDNA was then circularized by ligation with 200 units of T4 DNA ligase (New England Biolabs) for 20 hours at 15°C. Circularized cDNA was then used as template for PCR, using two primers corresponding to the mPH-30β sequence. One primer was nested to the gene-specific primer used for the reverse transcription (GGAATCATGAGGTCTCTTGA, corresponding to nt # 253-272). The other primer (GAGATTGTAGGTCACCTGTG, corresponding to nt # 146-165) was designed to span the ligation junction. The PCR product was cloned into pCRII and sequenced as described above. Two different clones were sequenced.

Northern blot analysis

A DNA probe was prepared as described above, with the exception that a PCR product spanning nt # 146-2058 was used as template for random primed labelling; 1×10^6 cpm/ml of this probe were used per blot. Northern blots containing 20 μg total RNA per lane from mouse brain, liver, spleen, unfractionated male germ cells, pachytene spermatocytes, and round spermatids were generously provided by Stuart Moss (University of Pennsylvania). Prehybridization, hybridization, and washing were performed as described (Carrera et al., 1994).

Reverse transcription-PCR

RNA from adult mouse testis, day 17 post-natal mouse testis, pachytene spermatids, and round spermatids was generously provided by Stuart Moss. First strand cDNA was generated by reverse transcription as previously described (Temeles et al., 1994). PCR was performed using primers designed to match the sequence of the mPH-30β clone (to generate a product of 305 bp) in a manner identical to that described above with the exception of the amplification program used (one cycle of 94°C for 60 seconds; 30 cycles of 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds; and a final extension cycle of 72°C for 5 minutes). PCR products were analyzed by agarose gel electrophoresis according to standard protocols (Sambrook et al., 1989).

RESULTS

Effects of divalent cations on sperm-egg binding and fusion

Many integrins interact with their ligands in a divalent cation-dependent manner. In addition, several integrins show preferential activity in the presence of one divalent cation versus another. The Ca^{2+} dependence of mouse and hamster fertilization has been examined (Miyamoto and Ishibashi, 1975; Fraser, 1987; Fujimoto et al., 1994; Yanagimachi, 1987). The experimental design of these studies in mouse, however, would not permit an analysis of the individual cation requirements for both sperm-egg binding and fusion, since Mg^{2+} was present with Ca^{2+}; thus, one cannot conclude that Ca^{2+} is required for sperm-egg membrane interactions from these studies as they do not eliminate a role for Mg^{2+}. Previous studies of sperm function in guinea pig, hamster, and human did examine the role of individual divalent cations but focussed exclusively on fusion and not on binding (Yanagimachi, 1978). To extend these studies, we examined the dependence of IVF using ZP-free mouse eggs on three different divalent cations, Ca^{2+}, Mg^{2+}, and Mn^{2+}, since integrins utilize these three cations. Our goal was to determine if the interaction(s) of the gamete plasma membranes depended on the presence of divalent cations, and if this interaction(s) occurred preferentially in the presence of specific divalent cations at specific concentrations.

Three different endpoints of sperm-egg interaction were examined in the IVF experiments presented throughout this study. Two of these, the percentage of eggs fertilized and the average number of sperm fused per egg, are measures of sperm-egg fusion. The percentage of eggs fertilized represents the percentage of eggs penetrated with at least one sperm; this value does not distinguish between monospermic and polyspermic eggs. The average number of sperm fused per egg reflects both monospermy and polyspermy. It should be noted that we obtained very low levels of polyspermy due to the low concentrations of sperm used (see Table 1 for details). A third endpoint, the average of the number of sperm bound per egg, was also determined; this value included sperm that had fused with the egg, based upon the assumption that these sperm were bound to the egg surface prior to fusion.

Sperm binding was supported by each of the three cations (Ca^{2+}, Mg^{2+}, and Mn^{2+}) at three concentrations (0.1, 1.0 and 5.0 mM) (Fig. 1A, open bars). In the absence of Ca^{2+}, Mg^{2+}, and Mn^{2+} (medium to which these divalent cations were not added), sperm motility was drastically reduced, and this resulted in a decrease in binding and fusion that could not be clearly attributed to a failure in the recognition of a sperm ligand by an egg receptor. In all of the concentrations of individual divalent cations added, sperm motility was similar (>80% motile sperm). While sperm binding was somewhat variable at the different divalent cation concentrations, sperm binding did occur in the presence of each of the three divalent cations at 0.1, 1.0, and 5.0 mM (Fig. 1A, open bars). With the exception of the experiments carried out in the presence of 0.1 mM Mg^{2+}, none of these slight differences in binding were statistically significant ($P>0.05$; *t*-test) as compared to the Whitten's medium control (which has 1.2 mM Mg^{2+} and 2.4 mM Ca^{2+}; Whitten, 1971). For each individual divalent cation tested, binding observed in 0.1 mM was reduced as compared to that in 1.0 and 5.0 mM. As previously reported (Yanagimachi, 1978), incubation in 1.0 and 5.0 mM Mn^{2+} occasionally resulted in lysis of a portion (10-40%) of the eggs.

In contrast to sperm binding, sperm-egg fusion only occurred in the presence of Ca^{2+}, which was necessary and sufficient (Fig. 1A, solid bars; and Fig. 1B), and with an optimum around 2.5 mM (Fig. 2A and B, solid bars). Fertilization was slightly more efficient than in our control medium, since a statistically significant higher percentage of eggs were fertilized in 2.5 mM Ca^{2+} than eggs in Whitten's medium (Fig. 2A; $P<0.05$). With the exception of inseminations performed in divalent cation-deficient medium (0 mM, Fig. 2B), sperm binding in these samples was comparable, as none of these treatments resulted in a statistically significant change in sperm binding as compared to the Whitten's medium control ($P>0.05$).

Effects of RGD peptides on sperm-egg binding and fusion

Many members of the integrin family recognize the tripeptide RGD in the cell recognition region of their ligands and RGD peptides disrupt these interactions (Hynes, 1992; Haas and Plow, 1994). To determine if mouse sperm-egg membrane interactions utilized an RGD-dependent egg receptor molecule, eggs were initially incubated with and then inseminated in the presence of various RGD peptides (GRGDTP, cRGD, and GRGDSP; GRGESP as negative control). These fertilization

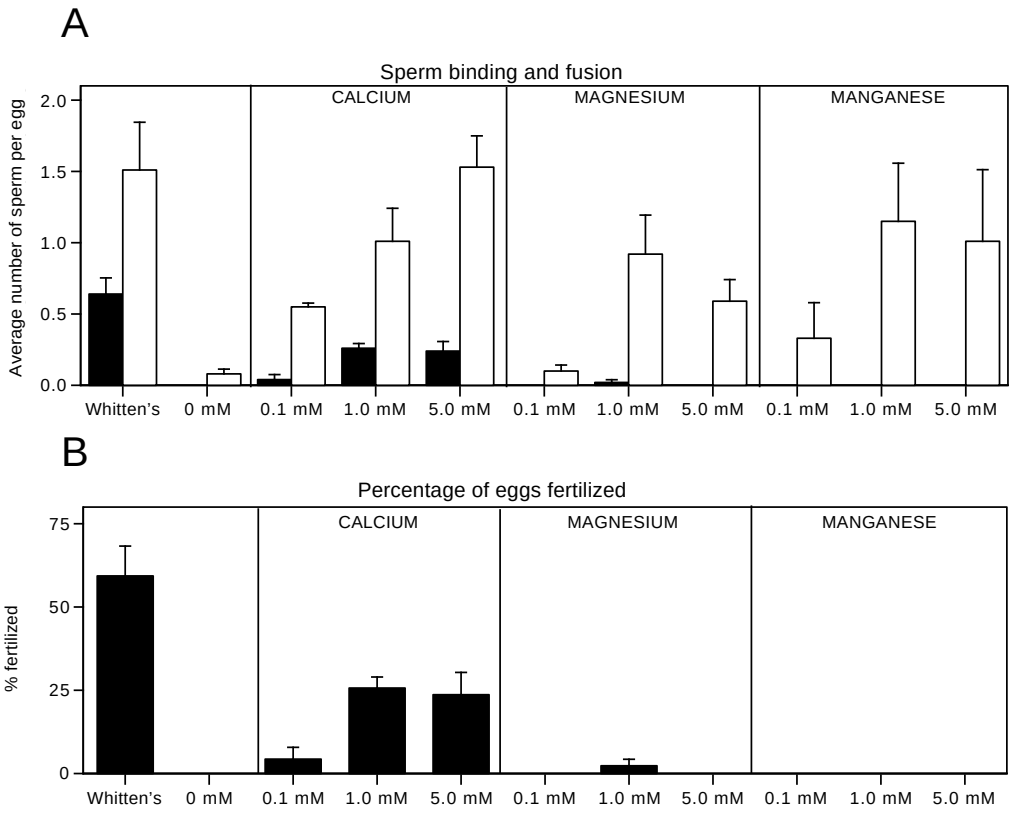


Fig. 1. Effects of divalent cations on sperm-egg binding and fusion. ZP-free mouse eggs were inseminated in divalent cation-deficient medium (0 mM $Ca^{2+}/Mg^{2+}/Mn^{2+}$), or in medium containing 0.1, 1.0, or 5.0 mM Ca^{2+}, Mg^{2+}, or Mn^{2+}. Inseminations were performed using 10,000-20,000 sperm/ml. Eggs were inseminated in Whitten's medium as a positive control (see Materials and Methods). (A) The average number of sperm fused (solid bars) and bound (open bars) per egg. (B) The percentage of eggs fertilized. The results here represent the average ± s.e.m. of three experiments per divalent cation concentration and a total of approximately 60 eggs fertilized per concentration of each divalent cation.

experiments were performed with low concentrations of sperm (5,000-8,000/ml) in order to bias conditions to detect inhibition. Under these conditions, control fertilizations (no peptide) resulted in fertilization of 25-50% of the eggs. Parallel inseminations were performed with higher concentrations of sperm (15,000-25,000) to ascertain egg quality and verify that higher levels of fertilization (>50%) could be obtained. Under these conditions of higher sperm concentration, RGD peptides had very little effect on fertilization (data not shown). In inseminations performed with the lower sperm concentrations, 2.5 mM RGD peptides moderately decreased the percentage of eggs fertilized (Fig. 3A) and number of sperm fused per egg (Fig. 3B, solid bars), indicating that RGD peptides perturbed sperm-egg fusion. Sperm-egg fusion was reduced in the presence of GRGDTP (58% reduction in percentage of eggs fertilized) and cRGD (52% reduction of percentage of eggs fertilized). GRGDSP was relatively ineffective (30% reduction in percentage of eggs fertilized). A peptide containing the sequence RGE instead of RGD, a standard negative control for cell adhesion experiments (Pytela et al., 1987), did not have an inhibitory effect on fertilization (Fig. 3A,B). No RGD peptide caused a statistically significant (P<0.05) reduction in sperm binding (Fig. 3B, open bars). The inhibitory effect of the most effective peptide, GRGDTP, was shown to be concentration dependent, since in separate experiments, eggs inseminated in the presence of 1.0 and 0.5 mM GRGDTP showed levels of sperm binding and fusion nearly identical to those of the no peptide controls (data not shown).

Cloning and RNA expression of mouse PH-30β

The disintegrin domain of gpPH-30β does not have the sequence RGD as do most snake venom disintegrins (Gould et al., 1990) but instead has the sequence TDE. TDE-containing peptides at 0.5 mM completely inhibit sperm-egg fusion in the guinea pig (Myles et al., 1994). These results and our observations of only moderate inhibitory effects of RGD peptides raised two questions. First, do mouse sperm have a homologue of gpPH-30β and, if so, does the mouse homologue have a sequence other than RGD in its disintegrin domain? The mouse PH-30β (mPH-30β) homologue was cloned in order to address these questions.

The full-length nucleotide sequence is presented in Fig. 4 and the sequence has been deposited in GenBank (Accession number U16242). The sequence covers the region encoding all of the key functional domains of PH-30β, from the signal sequence to the cytoplasmic tail. Searches of the GenBank database revealed that this cDNA had highest homology to gpPH-30β (72% at the amino acid level); it is also a perfect match of a partial cDNA clone for mPH-30β that was recently deposited in GenBank and published (Wolfsberg et al., 1995). The cDNA for mPH-30β that we isolated also had homology to other members of this family of molecules: monkey tMDC I (Barker et al., 1994), monkey tMDC II (Perry et al., 1994), mouse cyritestin (Heinlein et al., 1994), mouse monocyte protein MS-2 (Yoshida et al., 1990), rat epididymal protein EAP-1 (Perry et al., 1992), and guinea pig PH-30α (Blobel et al., 1992; Wolfsberg et al., 1993). It should be noted that mPH-30β was not identical to the murine proteins of this molecular family (cyritestin and MS2).

mPH-30β cDNA had a polyadenylation signal and a poly(A) tail. One of the cDNA clones isolated in our library screening was found to have a poly(A) tail in a slightly different location. This was apparently due to the use of a degenerate polyadenylation signal (underlined in Fig. 4) that is sometimes used in

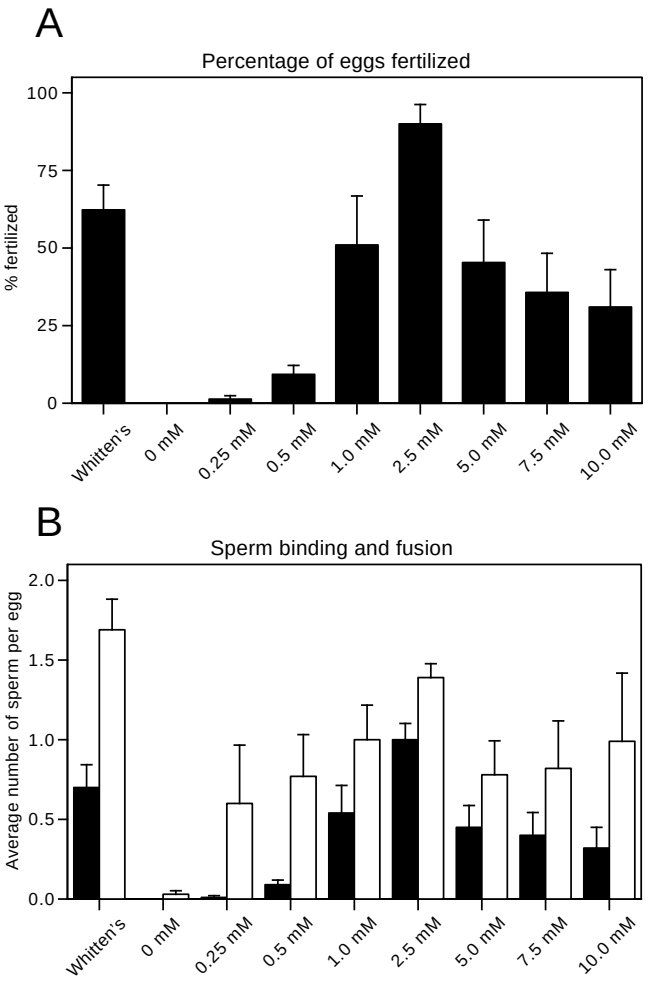


Fig. 2. Effects of Ca^{2+} on sperm-egg binding and fusion. ZP-free mouse eggs were inseminated in medium devoid of divalent cations (0 mM $Ca^{2+}/Mg^{2+}/Mn^{2+}$), or in medium containing 0.25-10.0 mM Ca^{2+}. Inseminations were performed using 10,000-20,000 sperm/ml. Eggs were inseminated in Whitten's medium as a positive control (see Materials and Methods). (A) The percentage of eggs fertilized. (B) The average number of sperm fused (solid bars) and bound (open bars) per egg. The results here represent the average ± s.e.m. of three experiments per divalent cation concentration and a total of approximately 60 eggs fertilized per concentration of Ca^{2+}.

germ cells (Moss et al., 1994). This cDNA, therefore, had a poly(A) tail that began 106 nt upstream from the poly(A) tail that follows the consensus polyadenylation signal (underlined and bold in Fig. 4). The deduced amino acid sequence from the mouse clone for PH-30β (labelled 'm') was 55% identical and 72% homologous to that of gpPH-30β (Fig. 5, labelled 'gp'). All of the cysteines, which are believed to be critical for the structure of the molecule, were conserved between the mouse and guinea pig PH-30β, as they were conserved between guinea pig PH-30β and other disintegrins (see Fig. 1 in Wolfsberg et al., 1993). N-linked glycosylation sites (Asn-X-Ser/Thr) identified in guinea pig PH-30β (Blobel et al., 1992) were also conserved in mPH-30β. Also of note was the cell recognition region of the disintegrin domain. As mentioned above the tripeptide RGD, a recognition motif of several integrin receptors, is present in many snake disintegrins (Gould et al., 1990). In gpPH-30β, this region had the sequence TDE, and in mPH-30β the sequence was QDE.

mPH-30β transcripts were detected by northern blot analysis using RNA from unfractionated male germ cells, pachytene spermatocytes (lane P) and round spermatids (lane RS), but not in brain (lane B), liver (lane L), or spleen (lane S) (Fig. 6A). The transcipt was approximately 2.44 kb, which agrees with the size of the cDNA clone (2,546 bp). To determine more precisely when during spermatogenesis mPH-30β transcripts were expressed, RT-PCR was performed (Fig. 6B). PCR products were observed in reactions using total RNA from post-natal day 17 testis (lane 17), leptotene/zygotene spermatocytes (lane LZ), pachytene spermatocytes (lane P), round spermatids (lane RS), and condensing spermatids (lane CS) (Fig. 6B). These data are in agreement with the in situ hybridization results of Wolfsberg et al. (1995). Moreover, the diagnostic PCR product was detected in the adult testis, but not in brain or in control reactions from which template DNA was omitted (data not shown). As a positive control for template quality, brain RT was used for successful amplification with primers corresponding to actin (data not shown). As negative controls, identical RTs were performed in the absence of

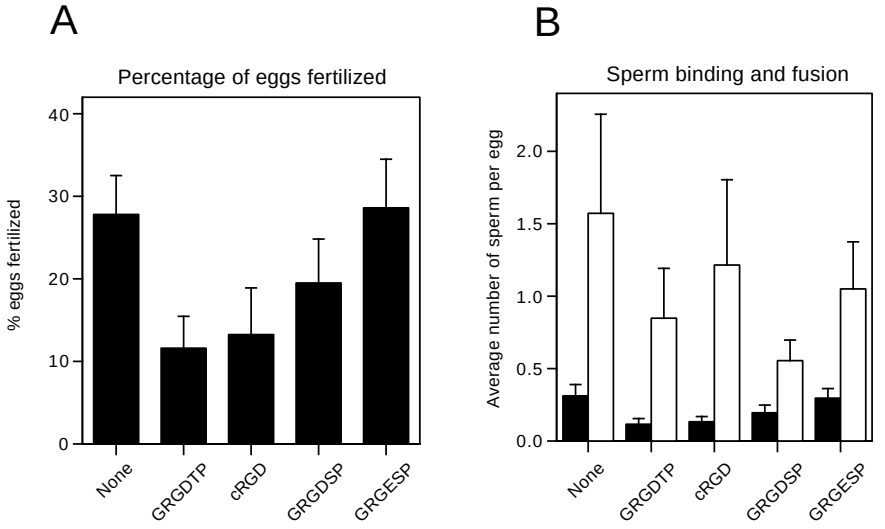


Fig. 3. Effects of RGD peptides on sperm-egg binding and fusion. ZP-free mouse eggs were incubated in 2.5 mM peptide (GRGDTP, cRGD = GPenGRGDSPCA, GRGDSP, or GRGESP; all from Gibco-BRL) for one hour prior to fertilization. Eggs were then inseminated with 5,000-8,000 sperm/ml in the presence of 2.5 mM peptide. (A) The percentage of eggs fertilized. (B) The average number of sperm fused (solid bars) and bound (open bars) per egg. The results here represent the average of 4-5 experiments per peptide ± s.e.m. and a total of 70-100 eggs fertilized per peptide.

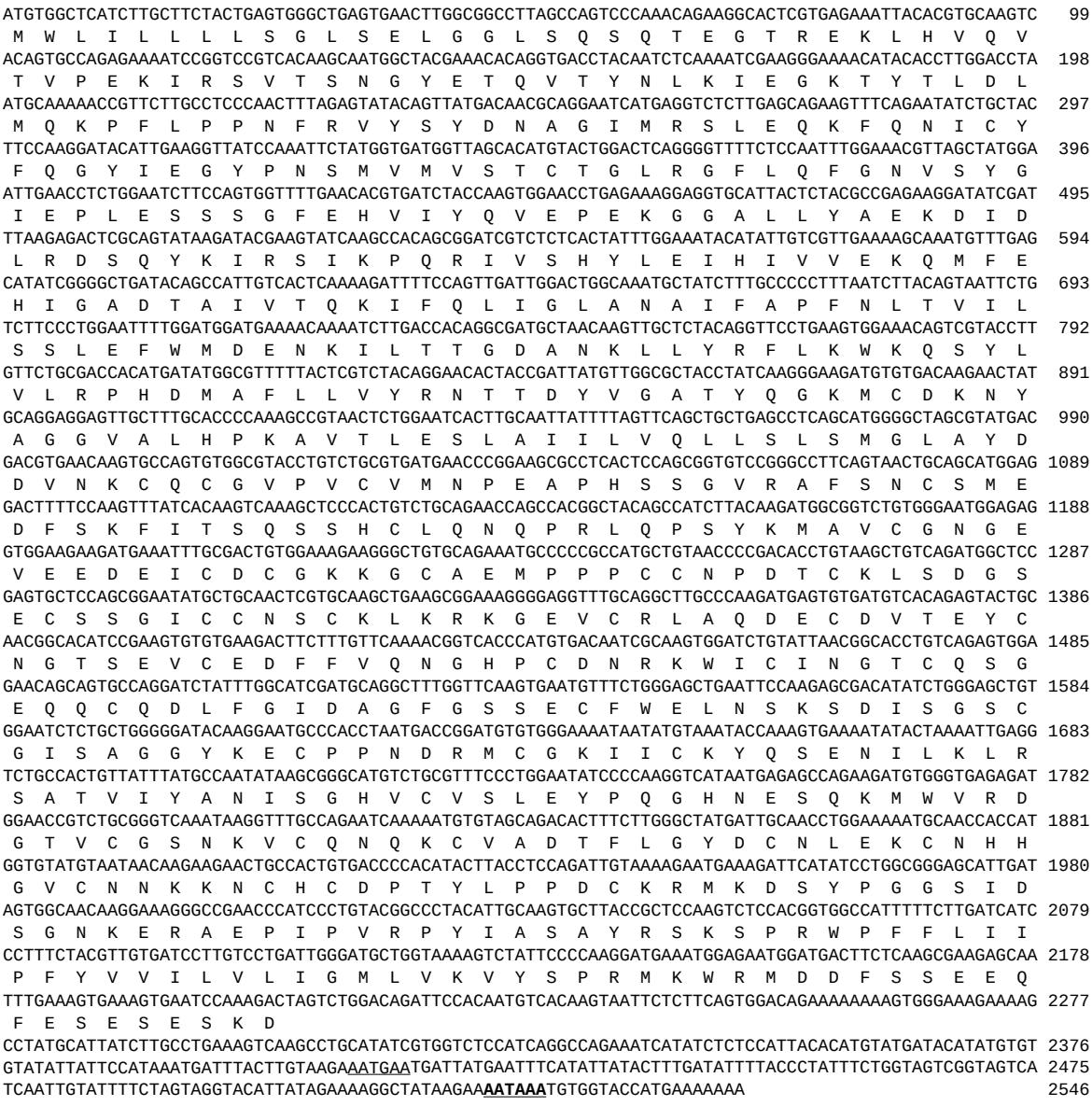


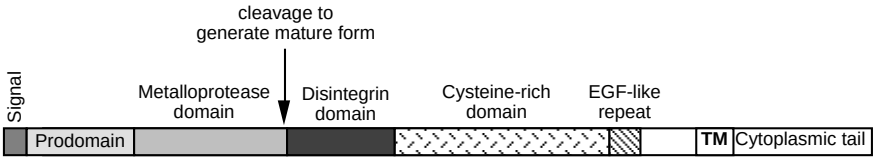
Fig. 4. Nucleotide and deduced amino acid sequence of mPH-30β. Nucleotide and deduced amino acid sequences are shown. The consensus polyadenylation signal is indicated in bold and underlined. A degenerate polyadenylation signal is underlined (see text for details).

reverse transcriptase; no PCR products were amplified from these control RT reactions, indicating that the PCR products were amplified specifically from reverse-transcribed cDNA (data not shown). These northern blot and RT-PCR results demonstrated that the RNA encoding mPH-30β was expressed during both the meiotic (leptotene/zygotene, pachytene spermatocytes, and testis from post-natal day 17 mice, which lack post-meiotic stages of germ cells; Bellvé et al., 1977) and post-meiotic (round and condensing spermatids) phases of spermatogenesis.

Effects of QDE peptides from mPH-30β on sperm egg binding and fusion

Peptides containing the tripeptide QDE sequence of mPH-30β's disintegrin domain were used in IVF experiments since mPH-30β was found to have a QDE sequence instead of RGD in its disintegrin domain. Linear CAQDEC (0.5 mM) reduced sperm binding (73%) and sperm-egg fusion (69% reduction in the percentage of eggs fertilized and 71% reduction in average number of sperm fused per egg when compared to no peptide controls) (Fig. 7A,B). It should be noted that the decrease in sperm binding was not solely due to a decrease in fertilization; eggs fertilized in the presence of linear CAQDEC demonstrated a reduced number of both fused sperm and non-fused sperm (data not shown). Cyclic peptides containing the sequence QDE, either CAQDEC cyclized by oxidation or covalently cyclized BrAcAQDEC (see Materials and Methods), did not have an inhibitory effect on sperm binding or fusion (data not shown).

The inhibitory effect of CAQDEC peptides was concentration dependent; no significant inhibition of binding or fusion was observed with 0.05, 0.1 and 0.25 mM CAQDEC (Fig. 7A,B). Higher concentrations of CAQDEC (1.0, 1.5 and 2.5 mM) did not result in a statistically significant increase in its

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                    PRODOMAIN
gp MLCLLLLLCGLASLGGPL-------KKYVENSGMQITVPEKIRSVKRGSVESEVSYKIVIENTTYIVNLVRKMFLPHDFQVYSYDSAGIMKPFEDYSQNF
   *+++**** **  ***+       +   *   +*+********      *+ *+* + **  ** + *+ *+*** * *****+****+ +*   **+
m  MWLILLLLSGLSELGGLSQSQTEGTR---EKLHVQVTVPEKIRSDTSNGYETQVTYNLKIEGKTYTLDLMQKPFLPPNFRVYSYDNAGIMRSLEQKFQNI

                                  METALLOPROTEASE
gp CYYQGHIEGFPTSLASISTCAGLRGLLQFETVSYGIEPLKSSIGFEHVIYPVKHDNEKSQ-YLKKSINVKNVVYKIKSIKSSVR-THYIEMHIIVEKNLY
   ** ** *** *+*++ +*** ****+***  ******** ** ******* *   +     *+ * * ++   ***+*** +   +**+*+**+***++
m  CYFQGYIEGYPNSMVIVSTCTGLRGFLQFGNVSYGIEPLESSSGFEHVIYQVEPEKGGALLYAEKDIDLRDSQYKIRSIKPQRIVSHYLEIHIVVEKQMF

gp KHMGGNTATVTEKIFQLVGLMNAFFSTLNLTVILASLELWIDENKIPTTGDVNELLHAFQKWKKSYLVLRPHDVAFLLVYRESPSYIGAIFQGMICNTSY
    *+*+ ** ** *****+**+**+*  +****** ***+*+*****+****+* **  * *** *********+******* +  *+**  ** +*  +*
m  EHIGADTAIVTQKIFQLIGLANAIFAPFNLTVILSSLEFWMDENKILTTGDANKLLYRFLKWKQSYLVLRPHDMAFLLVYRNTTDYVGATYQGKMCDKNY

                                                                                    DISINTEGRIN
gp GGGIALHSKTITLDSFGVILVQLLSVSMGIAYDNADLCRCRGAICLMSPEAVFSSGMKMFSNCSVKAFKLFTSSQVSQCLQNQPYLEPVYRSNPVCGNNR
   +**+*** * +**+*+++*******+***+*** +  * * +++*+*+***+ ***+++*****+  *  * +** * ****** * * *   +****
m  AGGVALHPKAVTLESLAIILVQLLSLSMGLAYDDVNKCQCGVPVCVMNPEAPHSSGVRAFSNCSMEDFSKFITSQSSHCLQNQPRLQPSY-KMAVCGNGE

                                                                                    CYSTEINE-RICH
gp VEQGEDCDCGSQEECQDT---CCDAATCRLKSTSRCAQGPCCNQCEFKTKGEVCRESTDECDLPEYCNGSSGACQEDLYVINGHRCANEEWICMNGRCLS
   **  * ****    * +    ** + ** *   * *  *+***+* +* ******  +****+ *****+* +* **+ * *** * *  ***+** * *
m  VEEDEICDCGKKG-CAEMPPPCCNPDTCKLSDGSECSSGICCNSCKLKRKGEVCRLAQDECDVTEYCNGTSEVC-EDFFVQNGHPCDNRKWICINGTCQS

gp GKAQVQETFGTEMEMGSVDCFEQLNTKNDITGNCGILSPGNYKACGASNWKCGKLICSYDKSEILRNKEGMTIYANISGHICVSIEYPPGHAKSALMWVR
   *  * *+ ** ++ +** +**  **+*+**+*+*** *+* ** *+++   ***+** *    **+ + +  ********+***+*** **  *  ****
m  GEQQCQDLFGIDAGFGSSECFWELNSKSDISGSCGI-SAGGYKECPPNDRMCGKIICKYQSENILKLRSATVIYANISGHVCVSLEYPQGHNESQKMWVR

                                  EGF-LIKE REPEAT                                                   TM
gp DGTVCGPSEVCRQQQCVSSSYLGYDCTPATCSDHGVCNNKRHCHCNPTYVPPNCETQ-DSTKPGGSVDSGNL-RYEPIPETYFVEGAYHTKSRKWPFFLI
   ****** + ** +* **  + *****++  *+ *******++*** ***+** *    **+ ****+****  * ****    +  ** +**  ******
m  DGTVCGSNKVCQNQKCVADTFLGYDCNLEKCNHHGVCNNKKNCHCDPTYLPPDCKRMKDSY-PGGSIDSGNKERAEPIPVRPYIASAYRSKSPRWPFFLI

                    CYTOPLASMIC
gp IPFFVIFSVLVATVVKVYYQKKKWKTEDYANDENIESESEPKSSKVSSK
   *** *++ **++ +****+   **  +*  ++*++***** *
m  IPFYVVILVLIGMLVKVYSPRMKWRMDDFSSEEQFESESESKD
```

Fig. 5. Comparison of deduced amino acid sequence of gpPH-30β and mPH-30β. The upper portion of the figure shows the domains of gpPH-30β (adapted from Wolfsberg et al., 1993). The lower portion of the figure shows the deduced amino acid sequences of guinea pig (gp) and mouse (m) PH-30β. The domains of gpPH-30β are labelled, with the first amino acid of the indicated domain being underlined. An asterisk (*) indicates an amino acid identity; a plus sign (+) indicates a substitution of a conserved amino acid. The deduced amino acid sequence from the mouse clone for PH-30β (labelled 'm') is 55% identical to gpPH-30β (labelled 'gp'). Amino acid homology, based on identical amino acids and conserved amino acid substitutions, is 72%. All of the cysteines, which are believed to be critical for the structure of the molecule, are conserved between the mouse and guinea pig PH-30β, as they are conserved between guinea pig PH-30β and other disintegrins (Wolfsberg et al., 1993). Also of note is the cell recognition region of the disintegrin domain. The tripeptide RGD, a recognition motif of several integrin receptors, is present in many snake disintegrins. In gpPH-30β, this region has the sequence TDE; mouse PH-30β has the sequence QDE (bold).

inhibitory effect (data not shown). The linear control peptide (CDEQAC, 0.5 mM) did not have a significant inhibitory effect, although the percentage of eggs fertilized and average number of sperm fused and bound per egg were slightly lower than it was in the no peptide controls (Fig. 7A,B). Higher concentrations of the control peptide (1.0, 1.5, and 2.5 mM) caused reductions in sperm binding and fusion that were similar to the effects observed with the same higher concentrations of CAQDEC, suggesting that the effects of higher concentrations of peptide were non-specific (data not shown). There are at least three possible explanations for this. First, the decrease in binding and fusion could be due to chelation of divalent cations by these acidic peptides since we observe reduced levels of sperm binding and fusion in low concentrations of Ca^{2+}, Mg^{2+}, and Mn^{2+} (Fig. 1A,B). Second, it could be due to a contaminant that interferes with normal sperm and/or egg function that reaches critical levels in the higher concentrations of peptide. Finally, the presence of the Asp and Glu residues in succession could have a slight inhibitory effect. The control peptide CDEQAC was designed so that an acidic residue would

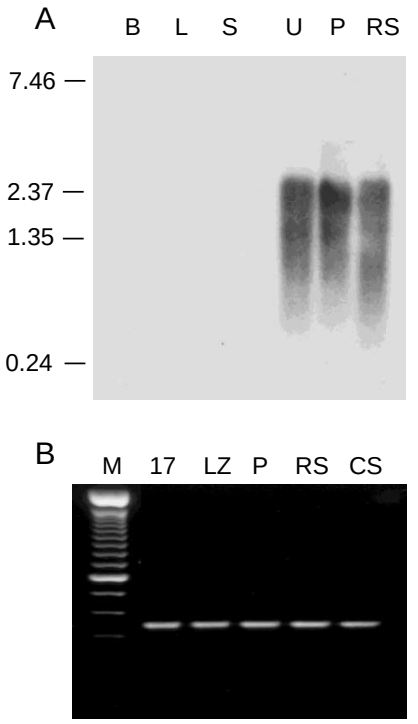

Fig. 6. Northern blot and reverse transcription-PCR detection of mPH-30β RNA expression. (A) A northern blot probed with an mPH-30β DNA probe; each lane contain 20 μg total RNA from brain (lane B), liver (lane L), spleen (lane S), unfractionated male germ cells (lane U), pachytene spermatocytes (lane P), and round spermatids (lane RS). (B) The PCR products amplified with two primers designed to match the mPH-30β sequence; these primers should amplify a 305 bp product. These products were amplified from cDNA reverse-transcribed from post-natal day 17 pup testis (lane 17), leptotene/zygotene spermatocytes (lane LZ), pachytene spermatocytes (lane P), round spermatids (lane R), and condensing spermatids (lane CS). M, 100 base pair ladder. The intense band in the middle of the gel corresponds to 600 bp.

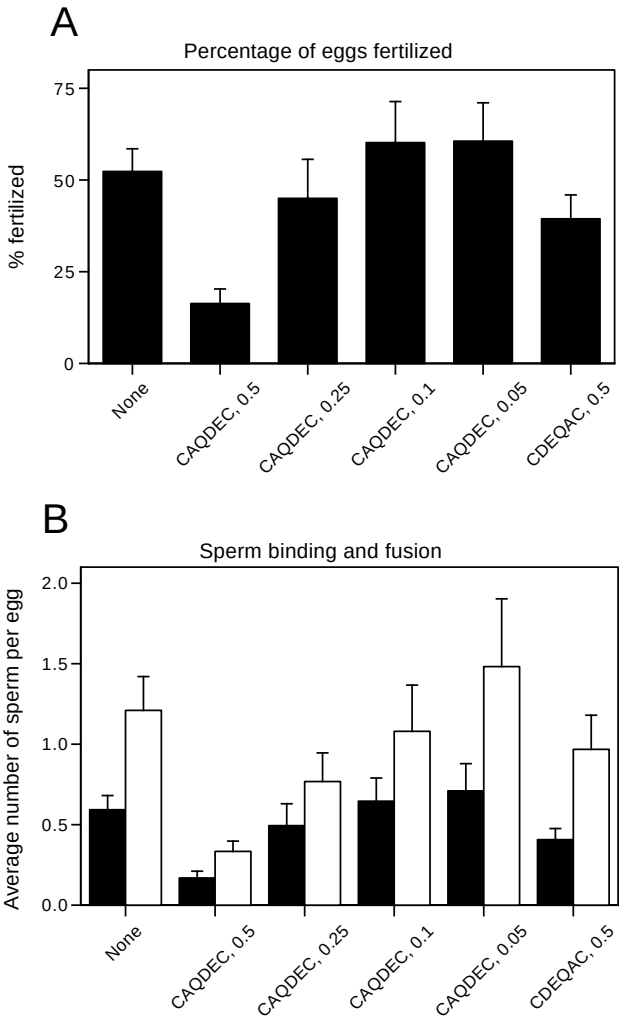

Fig. 7. Effect of QDE peptides on sperm-egg binding and fusion. ZP-free mouse eggs were incubated in the indicated concentration (mM) of peptide (CAQDEC or CDEQAC) for one hour prior to insemination. Eggs were then inseminated with 10,000-20,000 sperm/ml in the presence of peptide. (A) The percentage of eggs fertilized. (B) The average number of sperm fused (solid bars) and bound (open bars) per egg. The results here represent the average of 12 experiments (none, 0.5 mM CAQDEC, 0.5 mM CDEQAC) or 5 experiments (0.25, 0.1, and 0.05 mM CAQDEC) per peptide ± s.e.m. and a total of 100-250 eggs fertilized per peptide.

not be in the third position of the tripeptide sequence as is the case of RGD, TDE, and QDE; however, the presence of two adjacent acidic residues could produce an inhibitory effect. Nevertheless, statistical analysis showed that the levels of fertilization, sperm binding, and sperm fusion observed in eggs with 0.5 mM of control peptide were not different from those levels in eggs in the no peptide control, and the levels observed in the 0.5 mM CAQDEC peptide were significantly different from those of the no peptide control and 0.5 mM control peptide.

DISCUSSION

The work presented here on the divalent cation dependence and RGD peptide inhibition of fertilization in the mouse was undertaken to determine if integrins are involved in mammalian fertilization as previously suggested for hamster (Bronson and Fusi, 1990) and guinea pig (Blobel et al., 1992). Integrins have divalent cation binding sites, and recent results suggest that displacement of divalent cations from these sites by ligand molecules is an important step in ligand binding (D'Souza et al., 1994). While there are examples of integrins interacting with specific ligands in a divalent cation-independent manner (Vogel et al., 1993; Lallier and Bronner-Fraser, 1991), in general divalent cations are required for integrin function. Divalent cation-dependent integrins often show a preference for one divalent cation over another. For example, $\alpha_v\beta_1$ binds fibronectin more efficiently in Mg^{2+} than in Ca^{2+}; $\alpha_v\beta_3$ binds vitronectin more efficiently in Ca^{2+} than Mg^{2+} (Kirchhofer et al., 1991). Data such as these prompted us to determine the divalent cation dependence of gamete membrane interaction(s) at the level of both sperm binding and sperm fusion with ZP-free mouse eggs. Our results show that binding can occur in the presence of Ca^{2+}, Mg^{2+}, or Mn^{2+} (Fig. 1A), but fusion only occurs in the presence of Ca^{2+} (Fig. 1A,B). In addition, fertilization in 2.5 mM Ca^{2+} appears to be slightly more efficient

than fertilization in Whitten's medium, which contains 2.4 mM Ca^{2+} and 1.2 mM Mg^{2+} (Fig. 2A). Previous studies of mouse fertilization suggest that high concentrations of Ca^{2+} (7.2 mM) decrease the efficiency of fusion (Fraser, 1987), in agreement with our finding that 2.5 mM Ca^{2+} is optimal. Previous studies of fusion of guinea pig, human, and hamster sperm with eggs demonstrate that high concentrations (up to 5 mM) of Mg^{2+}, Sr^{2+}, Mn^{2+}, and Ba^{2+} could compensate for the absence of Ca^{2+} to variable extents (Yanagimachi, 1978). In contrast, we find that neither 5 mM Mg^{2+} nor 5 mM Mn^{2+} supports fusion of mouse sperm with mouse eggs.

To our knowledge, the work presented here is the first demonstration that mouse fertilization can occur with Ca^{2+} as the only divalent cation. The data presented here show that Ca^{2+} is sufficient to support binding and is necessary for fusion in the mouse. This suggests that a Ca^{2+}-dependent cell adhesion molecule (e.g. an integrin, a cadherin, or a novel molecule) may mediate both sperm binding and fusion. Another possibility is that binding occurs via a receptor that utilizes either Ca^{2+}, Mg^{2+}, or Mn^{2+}, and then fusion occurs via a receptor that requires Ca^{2+}. Sperm that have bound to eggs in 1.0 mM Mg^{2+} are able to undergo fusion when the eggs are transferred to medium containing 2.5 mM Ca^{2+} (data not shown); however, this does not rule out the possibility that sperm are transferred to a Ca^{2+}-dependent cell adhesion molecule that supports both binding and fusion.

We also examined the RGD peptide inhibition of mouse sperm-egg membrane interactions to elucidate further if integrins participate in fertilization. RGD peptides specifically compete for ligand binding sites due to the presence of an RGD tripeptide in several integrin ligands. While not all integrin-ligand interactions are RGD-dependent (Garcia-Pardo et al., 1990; Gehlsen et al., 1992; Vogel et al., 1993), there are several integrins that do recognize the RGD sequence (see Table 1 in Hynes, 1992). In addition, disintegrins bind to integrins via RGD tripeptides (Gould et al., 1990; Haas and Plow, 1994).

The binding and fusion of human and hamster sperm to hamster eggs has been shown to be inhibited by RGD peptides (Bronson and Fusi, 1990), suggesting that RGD-dependent integrins on hamster eggs participate in these processes. The results of our studies of mouse fertilization using RGD peptides share some similarities with those of Bronson and Fusi (1990); in agreement with that study, we find that RGD peptides can inhibit mouse fertilization, and that GRGDTP is the most effective peptide. However, our results differ significantly in several aspects from those of Bronson and Fusi. First, our studies in the mouse show that the effect of RGD peptides is only observed when IVF is performed using low sperm concentrations (5,000-8,000 sperm/ml). It should be noted that this is similar to the previous finding that a particular monoclonal antibody inhibits the attachment of neural crest cells to low concentrations of laminin (1 μg/ml) but not to high concentrations (10 μg/ml) (Lallier and Bronner-Fraser, 1991). Thus, in these two examples, adhesion could only be inhibited when limited amounts of ligand (e.g. laminin or the ligand on sperm) are presented to the target cell. In contrast to our experiments, Bronson and Fusi's studies of fertilization of ZP-free hamster eggs were performed using 6.7×10^6 sperm/ml. Second, we find that we need a much higher concentration of RGD peptide than was used in the previous study (Bronson and Fusi, 1990); 30 μM of GRGDTP is reported to inhibit completely fusion of human and hamster sperm with hamster eggs. To achieve moderate levels of inhibition of fusion of mouse sperm with mouse eggs (58%), we need 2.5 mM of GRGDTP. It should be noted that this concentration is not excessive, as standard protocols for studies of cell adhesion routinely use 0.1-3 mM of RGD peptides (Pytela et al., 1987). Third, as mentioned above, we observe a very different extent of inhibition. In the mouse, we do not observe a statistically significant reduction in sperm binding in the presence of RGD peptides and we observe up to 58% inhibition of sperm-egg fusion; Bronson and Fusi observe complete inhibition of binding to and fusion with hamster eggs. Finally, it should be noted that the animal models that we and Bronson and Fusi use are different. One explanation for these striking differences between their results and our results could simply be due to differences in the animal models used. Notable differences exist between the requirements for sperm fusion with hamster and mouse eggs, namely, the ability of other divalent cations to substitute for calcium (see above) and the ability to be inhibited by RGD peptides. Moreover, it should be noted that hamster eggs are promiscuous in their ability to fuse with sperm from other mammalian species, including sperm from other rodents, primates, and cetaceans (Yanagimachi, 1994). This indiscriminate fusion may be mediated by a unique receptor(s) on the hamster egg surface that participates in binding and/or fusion of many different species' sperm; this receptor(s) could be extremely sensitive to inhibition by RGD peptides.

The presence of a QDE sequence instead of RGD in the mPH-30β disintegrin domain likely accounts for the partial inhibitory effect of RGD peptides on mouse fertilization. CAQDEC peptide at 0.5 mM reduces sperm binding, consequently reducing the incidence of sperm-egg fusion (Fig. 7A,B). Thus, our results provide the first evidence that mPH-30 may be bifunctional, namely, that mPH-30 participates in both binding and fusion. This has been suggested previously for gpPH-30, based primarily on the cDNA sequence that reveals the presence of a disintegrin domain and a viral fusion peptide (Blobel et al., 1992). However, work with gpPH-30 and other molecules implicated in sperm-egg fusion only demonstrates a role for these molecules in fusion; no decrease in sperm binding is observed in these studies (Saling et al., 1985; Primakoff et al., 1987; Rochwerger et al., 1992; Myles, 1993).

Our results are similar but not identical to the finding that TDE peptides inhibit fusion of guinea pig sperm with in vitro matured guinea pig oocytes (Myles et al., 1993). One difference is that 0.5 mM TDE peptide completely inhibits fertilization of guinea pig eggs (obtained following maturation in vitro), while a 70% inhibition is obtained with the same amount of QDE peptide in the mouse. It is possible that there are multiple molecules or sites on molecules that participate in mouse fertilization, such that inhibiting only the receptor(s) that recognizes QDE would not necessarily have a completely inhibitory effect; other integrin ligands have multiple binding sites for their receptors (Smith et al., 1990; Nagai et al., 1991). Likewise, mPH-30β could have multiple egg recognition sites, or mouse sperm could have additional ligands for an egg receptor that participate in sperm-egg binding and/or fusion. A second difference is that linear and cyclized TDE peptides have inhibitory activity in the guinea pig; in contrast, only linear peptides inhibit mouse fertilization, and peptides cyclized covalently or by oxidation have no effect (data not shown).

There are at least two possible explanations for these results. One possibility is that the receptor on guinea pig eggs could have a different conformation from the receptor on mouse eggs, such that the guinea pig receptor is recognized by a cyclic peptide and the mouse receptor is recognized more effectively by a linear peptide. An additional possibility is that Myles et al. (1994) had enough linear peptide in their cyclized peptide preparation (oxidized in water) to exert an inhibitory effect. In our hands, <30% of the peptide is oxidized in water.

This raises the issue of a difference in the apparent effect of RGD peptides and QDE peptides on fertilization. RGD peptides at 2.5 mM inhibit fertilization when conditions do not heavily favor fertilization (i.e. low sperm concentration), although they do not cause a statistically significant decrease in sperm binding. In contrast, QDE peptides at 0.5 mM inhibit fertilization when higher sperm concentrations are used, and these peptides decrease both sperm binding and fusion. This difference may reflect the possibility that the RGD peptides may be poor analogs of the site recognized by the egg receptor. Alternatively, two different receptors on the egg surface may be involved. For example, QDE peptides could inhibit one receptor that is involved in sperm binding while RGD peptides inhibit a completely different receptor that participates in fusion. It should be noted that membrane fusion is a complicated multi-step process (White, 1992). In addition, certain types of cell adhesion may occur via multi-step processes. For example, leukocyte-endothelium attachment and adhesion utilizes a variety of cell adhesion molecules in a step-wise manner (Lasky, 1992; Rosen and Bertozzi, 1994). Therefore, it is likely that sperm-egg binding and fusion would utilize multiple molecules and/or multiple sites on molecules. Nevertheless, we have not been able to detect an increased inhibitory effect with a combination of QDE and RGD peptides (data not shown).

The results presented here suggest that the disintegrin domain of mPH-30β serves as a ligand for a mouse egg receptor. A candidate receptor could be an egg integrin; mouse eggs express α_6, α_v, and β_1 integrin subunits at their surface (Tarone et al., 1993; Hierck et al., 1993; Evans et al., 1995). However, it has not been demonstrated that an egg integrin serves as the receptor for mPH-30β. First, unlike the results of Bronson and Fusi (1990), our data with RGD peptides do not strongly suggest the involvement of an RGD-dependent integrin in mouse fertilization. Second, we have been unable to inhibit sperm binding or fusion with antibodies to α_6, α_v, and β_1 (data not shown). Third, screens of phage display libraries for peptide ligands for integrins have failed to identify a QDE or TDE sequence that binds to any integrin (O'Neill et al., 1992; Koivunen et al., 1993, 1995). Therefore, resolution of the role of egg integrins in sperm-egg interactions awaits the identification of the egg receptor(s) for PH-30.

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Recently, Almeida et al. (*Cell* **81**, 1095-1104, 1995) provided evidence implicating the integrin $\alpha_6\beta_1$ on the mouse egg surface in binding sperm to the egg's plasma membrane.