Potential role of αv and $\beta 1$ integrins as oocyte adhesion molecules during fertilization in pigs

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Integrin molecules are cell adhesion molecules that are thought to be involved in sperm–oocyte interaction in rodents and humans. The objective of this study was to evaluate whether integrin molecules were present on the surface of pig oocytes, consistent with involvement in sperm–oocyte interaction in this species. Immunocytochemistry and confocal microscopy were used to evaluate the presence of $\beta 1$, and $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$ and αv integrin subunits on the plasma membrane of pig oocytes. The $\beta 1$ and αv integrin subunits were present consistently at the surface of pig oocytes; however, the remaining α integrin subunits evaluated were not routinely detected. The antibodies to the $\beta 1$ and αv integrin subunits recognized appropriately sized protein bands on western blots of partially purified oocyte plasma membrane. These two antibodies also recognized oocyte plasma membrane protein isolated from a sperm plasma membrane affinity column. Sperm plasma membrane proteins of 137 and 93 kDa appeared to be the ligands for the $\beta 1$ integrin subunit as revealed by a western sandwich blot. Antibody to an extracellular domain of the $\beta 1$ integrin subunit reduced pig sperm–oocyte binding ($P < 0.05$), also indicating an assisting role for a $\beta 1$ oocyte integrin subunit in sperm–oocyte interaction in pigs. These results are consistent with an $\alpha v\beta 1$ pig oocyte integrin interacting with a ligand on the sperm plasma membrane during fertilization.

Introduction

Fertilization in mammals is a multi-step process (Yanagimachi, 1994). After passage of acrosome-reacted spermatozoa through the zona pellucida, the fertilizing spermatozoon initially attaches to the oocyte plasma membrane via the inner acrosomal membrane (Koehler *et al.*, 1982; Talbot and Chacon, 1982). Subsequently, binding and fusion with the oocyte plasma membrane occur first with the equatorial segment of the spermatozoon followed by fusion in the post-acrosomal region (Yanagimachi, 1994). Molecules on the mammalian oocyte plasma membrane that interact with the sperm plasma membrane are not well understood, but an integrin heterodimer is proposed as one such receptor in rodents (Almeida *et al.*, 1995).

The discovery that the potential guinea-pig sperm binding molecule fertilin was a disintegrin has contributed significantly to the hypothesis that complementary binding molecules on the oocyte surface are integrins (Bibel *et al.*, 1992; Myles *et al.*, 1994; Wolfsberg *et al.*, 1995a; de Nadai *et al.*, 1996). The discovery of fertilins in other rodents and primates and reports of integrin molecules on the oocyte surface provided further support for the idea that one or more integrins may function as receptors for spermatozoa on the mammalian oocyte surface (Tarone *et al.*, 1993; Evans *et al.*, 1995; Wolfsberg *et al.*, 1995b; Hardy and Holland, 1996; McLaughlin *et al.*, 1997; Capmany

et al., 1998; Evans, 1999). Although integrins have been associated with the plasma membrane of rodent oocytes, the presence of these molecules on the surface of oocytes from the Order Artiodactyla has not been reported. The objective of this study was to examine whether integrins are present in pig oocytes, consistent with a function in sperm–oocyte plasma membrane interaction.

Materials and Methods

Antibodies

Rabbit polyclonal antibodies to the exterior portion of $\beta 1$ or $\alpha 5$ or to the C-terminal cytoplasmic domain of $\beta 1$, $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$ and αv were obtained from Chemicon Int., Inc. (Temecula, CA; catalogue numbers AB1937, AB1921, AB1952, AB1934, AB1944, AB1948, AB1924, AB1949 and AB1930). A rat monoclonal antibody to $\alpha 6$ (GoH3, catalogue number 33771A) was obtained from PharMingen (San Diego, CA). Crossreactivity of these antibodies (except AB1937 ($\beta 1$), AB1921 ($\alpha 5$) and AB1924 ($\alpha 4$)) with pig integrins was reported by the manufacturer. The reactivity of AB1921 with pig white blood cells or marrow cells or with the corresponding mouse cells (which should have been a positive control) was not conclusive. This finding prompted the use of the second antibody to the $\alpha 5$ subunit (C-terminal moiety), which had known crossreactivity with the pig

molecule. The crossreactivity of the $\alpha 6$ monoclonal antibody with pig $\alpha 6$ integrin subunit indicated by the supplier was verified in this laboratory with pig white blood cells, and the crossreactivity of AB1924 with pig white blood cells was demonstrated (data not shown).

Pig oocyte maturation

Pig ovaries were obtained approximately 5 min after exsanguination at the university abattoir. Oocytes were aspirated from antral follicles (2–3 mm diameter) into warm PBS (Table 1) and transported to the laboratory (30–37°C). Cumulus-intact oocytes were removed from the follicular aspirate and washed through three 100 μ l drops of PBS, two 100 μ l drops of HEPES–Tyrode's albumin lactate pyruvate (Table 1) and three 100 μ l drops of maturation medium (Table 1). A total of 10–15 oocytes were transferred to each 50 μ l drop of maturation medium and matured under oil for 24 h at 39°C in 5% CO₂ in air (Zheng *et al.*, 1992). Cumulus masses were removed from these partially matured oocytes during an approximate 5 min exposure to 1 mg hyaluronidase (500 U) ml⁻¹ in saline–BSA (0.9% (w/v) NaCl, 1 mg BSA ml⁻¹) followed by rinsing through three drops of saline–BSA. The zona pellucida was removed by brief exposure to 0.25 mg protease (3.75 U) ml⁻¹ in saline–BSA (catalogue number P5147, a type XIV protease from *Streptomyces griseus*; Sigma, St Louis, MO). When the zona pellucida showed some deformity, oocytes were transferred to saline–BSA and rinsed through at least two additional drops of saline–BSA until the zona pellucida was completely removed.

Antibody localization of integrin subunits on the oocyte surface

$\beta 1$ and $\alpha 5$ integrin subunits. Zona-free pig oocytes were incubated for 1 h with a 1:300 dilution of normal rabbit serum as a control or rabbit serum containing polyclonal antibodies to the $\beta 1$ or $\alpha 5$ integrin subunits. These oocytes were rinsed through three drops of saline–BSA to remove unbound serum

components followed by a 30 min incubation with a biotinylated goat anti-rabbit IgG (15 μ g ml⁻¹). Unbound IgG was removed during transfer of oocytes through three drops of saline–BSA. The oocytes were incubated for 30 min with 20 μ g tetramethylrhodamine isothiocyanate (TRITC)-tagged avidin, and rinsed three times to remove unbound avidin. The oocytes were fixed for 15 min in 3% (w/v) paraformaldehyde in PBS, rinsed, transferred to a drop of mounting medium (95% (v/v) glycerol, 5% (v/v) PBS containing 9.2 mmol *p*-phenylenediamine l⁻¹ and 15.38 mmol sodium azide l⁻¹, pH 8) and loaded into square glass capillary tubes (Vitro Dynamics, Inc., Rockaway, NJ; catalogue number 8320).

$\beta 1$, $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 6$ integrin subunits. Zona-free pig oocytes were fixed for 15 min in 3% (w/v) paraformaldehyde in PBS, rinsed through three drops of saline–BSA, and incubated with 2% (v/v) normal goat serum for 1 h to block non-specific binding. Oocytes were incubated with a 1:100 dilution of the rabbit polyclonal antisera to an integrin subunit or normal rabbit serum (all in the presence of 0.4% (v/v) normal goat serum). Oocytes were rinsed through three drops of saline–BSA and incubated for 30 min with fluorescein isothiocyanate (FITC)-conjugated second antibody (5 μ g goat anti-rabbit IgG ml⁻¹). Oocytes were rinsed through three drops of saline–BSA, transferred to mounting medium and loaded into square capillary tubes.

$\alpha 6$ subunit. The presence of the $\alpha 6$ subunit on fixed zona-free pig oocytes was evaluated using procedures similar to that described above. Normal rabbit serum (10% v/v) was used as the initial blocking serum, and oocytes were incubated with a 1:1000 dilution of rat monoclonal IgG to the $\alpha 6$ subunit or normal rat serum in the presence of 0.4% (v/v) normal rabbit serum. FITC-conjugated rabbit anti-rat IgG (31 μ g rabbit anti-rat IgG ml⁻¹) was used.

Visualization of bound antibody. Oocytes were scanned individually with a Zeiss LSM410 confocal microscope using the $\times 40$ objective to evaluate fluorescence on the plasma membrane (Wheeler *et al.*, 1996). The FITC fluorescence was observed using a 488 nm excitation wavelength, a 510 nm

Table 1. Composition of media used for *in vitro* maturation of pig oocytes

PBS	HEPES–TALP	Maturation medium
137 mmol NaCl l ⁻¹	114 mmol NaCl l ⁻¹	Medium 199 with
2.7 mmol KCl l ⁻¹	9.22 mmol HEPES l ⁻¹	L-glutamine, Earle's salts
8.4 mmol Na ₂ HPO ₄ ·7H ₂ O l ⁻¹	3.2 mmol KCl l ⁻¹	25 mmol HEPES l ⁻¹
1.5 mmol KH ₂ PO ₄ l ⁻¹	0.5 mmol MgCl ₂ ·6H ₂ O l ⁻¹	4.16 mmol NaHCO ₃ l ⁻¹
0.09 mmol CaCl ₂ l ⁻¹	0.655 mmol NaHCO ₃ l ⁻¹	3.05 mmol glucose l ⁻¹
5.5 mmol glucose l ⁻¹	2.0 mmol CaCl ₂ l ⁻¹	0.9 mmol sodium pyruvate l ⁻¹
0.32 mmol sodium pyruvate l ⁻¹	5.0 mmol glucose l ⁻¹	50 μ g gentamycin ml ⁻¹
0.05 mmol kanamycin SO ₄ l ⁻¹	13.2 mmol sodium lactate l ⁻¹	5 iu LH per 100 ml
4 mg BSA ml ⁻¹	0.1 mmol sodium pyruvate l ⁻¹	0.5 iu FSH per 100 ml
0.014 mmol phenol red l ⁻¹	0.168 mmol sodium penicillin l ⁻¹	
	3 mg BSA ml ⁻¹	
	0.028 mmol phenol red l ⁻¹	

TALP: Tyrode's albumin lactate pyruvate.

dichroic mirror and a 515 nm emission filter. The TRITC fluorescence was observed using a 568 nm excitation wavelength, a 580 nm dichroic mirror and a 590 nm emission filter. The binding of $\beta 1$, $\alpha 5$, αv and $\alpha 6$ antibodies and of respective controls was evaluated in a minimum of three replicates; in each replicate 5–15 oocytes were incubated with a single antibody. The binding of a second $\beta 1$ and a second $\alpha 5$ antibody (both recognizing the cytoplasmic portions of the integrin subunits) and of $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 4$ antibodies to fixed oocytes was examined on 10–15 zona-free pig oocytes in conjunction with the αv antibody as a positive control and normal rabbit serum as a negative control.

Western blotting

Partially purified pig oocyte plasma membrane was separated from an oocyte homogenate by density gradient centrifugation (Ash *et al.*, 1994b) after isolation of zona pellucida intact oocytes from pig ovaries and removal of zona pellucida fragments. Oocyte plasma membrane proteins (20 μ g per well) were solubilized under reducing conditions, separated on 10 cm $\times$ 12 cm tricine SDS polyacrylamide gels (Schagger and von Jagow, 1987) and transferred to Immobilon-P membrane (Millipore, Bedford, MA). Blots were incubated for 2 h with 5% (v/v) normal goat serum in Tris-buffered saline (TBS; 100 mmol Tris l^{-1} , 0.9% (w/v) saline, pH 7.5) containing 0.4% (v/v) Tween 20 (TTBS) to block non-specific binding sites. Blots were rinsed three times for 5 min each in TTBS. Individual lanes were incubated for 1 h with rabbit polyclonal antiserum to $\beta 1$ integrin subunit or normal rabbit serum (1:1000) or with rabbit polyclonal antiserum to αv integrin subunit or normal rabbit serum (1:300). Blots were again rinsed three times in TTBS followed by incubation with goat anti-rabbit IgG linked to horseradish peroxidase (Sigma, St Louis, MO). Unbound second antibody was removed by three successive rinses in TTBS and bound antibody was visualized with chemiluminescence (Renaissance, NEN, Boston, MA).

Western sandwich blots

Sperm plasma membrane proteins were solubilized under reducing conditions, separated by SDS-gel electrophoresis on Tris-glycine or Tris-tricine gels, and transferred to Immobilon-P membrane. Designated lanes were blocked for 1 h with TTBS followed by incubation with 40 μ g oocyte plasma membrane ml^{-1} for 2–3 h at room temperature in CTBS (TBS containing 16 mmol 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) l^{-1} and 2 mmol $CaCl_2$ l^{-1}). These lanes were rinsed, incubated with a 1:300 dilution of primary antibody (αv or $\beta 1$) in 1% (v/v) normal goat serum in TTBS containing 2 mmol $CaCl_2$ l^{-1} or a 1:500 dilution in TTBS containing 5–10% (v/v) normal goat serum and 2 mmol $CaCl_2$ l^{-1} with or without an additional blocking incubation in 5–10% (v/v) normal goat serum. Other lanes (not incubated with oocyte plasma membrane) were blocked with 5–10% (v/v) normal goat serum in TTBS for 1 h and incubated with 1:500 dilutions of normal rabbit serum,

or $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$ or αv antiserum, or a 1:300 dilution of $\beta 1$ antiserum in TTBS containing 1% (v/v) normal goat serum and 2 mmol $CaCl_2$ l^{-1} . Blot strips were rinsed and incubated for 1 h with a 1:80 000 dilution of peroxidase-labelled goat anti-rabbit antiserum, rinsed, incubated with chemiluminescence substrate threefold more dilute than recommended by the manufacturer, and bound antibody was observed on X-ray films. Lanes of sperm plasma membrane were also incubated with a 1:300 dilution of normal rat serum or $\alpha 6$ antibody as described for the polyclonal rabbit antibodies, followed by a peroxidase-labelled sheep anti-rat antibody.

Affinity chromatography

Isolated pig sperm plasma membrane proteins (Peterson *et al.*, 1980; Ash *et al.*, 1994a) were solubilized with CHAPS and attached to AminoLink gel (Pierce, Rockford, IL). Partially purified oocyte plasma membrane proteins (Ash *et al.*, 1994b) were incubated with the sperm plasma membrane affinity matrix for 3 h in CTBS. The unbound protein was removed by washing with 10 volumes of TTBS medium. Bound oocyte plasma membrane proteins were eluted with 0.5% (w/v) SDS in TBS and dialysed extensively against 10 mmol $NaHCO_3$ l^{-1} .

Slot blots

Approximately 1 μ g of affinity isolated oocyte plasma membrane was applied to each slot in a slot blot apparatus. Slot blots were probed essentially as described for western blots using a 1:1000 dilution of the polyclonal $\beta 1$ antiserum or normal rabbit serum and a 1:300 dilution of the polyclonal αv antiserum or normal rabbit serum. Each preparation of affinity-isolated oocyte plasma membrane was analysed twice.

In vitro sperm–oocyte interaction

Ejaculated pig spermatozoa were washed on a Percoll gradient and resuspended to a concentration of 4×10^6 spermatozoa ml^{-1} in Tris-buffered medium containing 10 mmol $CaCl_2$ l^{-1} (Berger and Horton, 1988). Spermatozoa were pre-incubated for 1 h at 39°C with 5% CO_2 before insemination of partially matured zona-free oocytes. Polyclonal antiserum ($\beta 1$ or $\alpha 5$ antibodies) was added to the oocytes before addition of the sperm suspension to provide a 1:300 final dilution of the serum. Gametes were co-incubated for 5 h at 39°C in 5% CO_2 in air, rinsed free of loosely attached spermatozoa and incubated overnight. Oocytes were placed on slides and compressed gently between slide and coverslip resting on ribbons of a mixture of vaseline and paraffin wax. After fixation and clearing for 3 days in 75% (v/v) methanol:25% (v/v) acetic acid, oocytes were stained with 1% (v/v) aceto-orcin and the stain was removed with 45% (v/v) acetic acid. The number of bound spermatozoa and number of penetrated spermatozoa were evaluated for each oocyte.

Since the polyclonal antibody to the extracellular domain of $\alpha 5$ did not bind to the oocyte surface, this antibody was chosen as the negative control. The number of spermatozoa bound per oocyte was evaluated in three separate replicates, in which the replicate was a random factor and the mean number of bound spermatozoa in each treatment within each replicate was weighted by the number of oocytes evaluated (SAS statistical programs, Cary, NC). The percentage of oocytes fertilized and the number of spermatozoa that penetrated per oocyte were similarly analysed.

Results

The antibody to the extracellular portion of the $\beta 1$ integrin subunit bound to the surface of unfixed oocytes (Fig. 1). Binding of this antibody to the oocyte surface showed variability among oocytes within each replicate. In some oocytes, the binding appeared uneven around the periphery of the oocyte, but the most typical pattern was a beaded one. Neither normal rabbit serum nor the polyclonal $\alpha 5$ antiserum bound to the periphery of unfixed oocytes. The αv antibody recognized the cytoplasmic tail of its integrin subunit and labelled the periphery of fixed pig oocytes (Fig. 2). Similarly, the binding of the αv antibody to the fixed oocytes appeared more evenly distributed than the binding of the $\beta 1$ antibody to unfixed oocytes. Both $\beta 1$ antibodies (one to the cytoplasmic portion and one to the extracellular domain) bound to fixed pig oocytes, similar to the pattern observed for the αv antibody. Fixed oocytes were not labelled by the rat monoclonal antibody to $\alpha 6$, by rabbit polyclonal antibodies to the cytoplasmic portions of the $\alpha 1$, $\alpha 2$ and $\alpha 5$ integrin subunits, or by normal rabbit or rat serum. Most of the fixed oocytes incubated with the polyclonal antibodies to cytoplasmic portions of $\alpha 3$ and $\alpha 4$

Fig. 1. Pig oocytes incubated with an antibody to the $\beta 1$ integrin subunit (a–c) or a control oocyte incubated with normal rabbit serum (d). Bound antibody was visualized with a biotinylated goat anti-rabbit second antibody followed by incubation with 20 μ g tetramethylrhodamine isothiocyanate (TRITC)-tagged avidin ml^{-1} .

integrin subunits were unlabelled, but a few (one-quarter or less) were very faintly labelled.

Western blotting

The oocyte plasma membrane preparations contained three bands (estimated M_r of 63, 87, and 126 kDa) recognized by the $\beta 1$ polyclonal antibody (Fig. 3). The αv antibody

Fig. 2. Pig oocytes incubated with an antibody to the αv integrin subunit (a–c) or a control oocyte incubated with normal rabbit serum (d). Bound antibody was visualized with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit second antibody.

Fig. 3. Western blot of pig oocyte plasma membrane proteins. Lane 1 was probed with normal rabbit serum and lane 2 was probed with the $\beta 1$ integrin subunit antibody. The M_r standards are indicated on the left-hand side.

recognized a single band with an estimated M_r of 124 kDa (Fig. 4).

Slot blots

The affinity-isolated oocyte plasma membrane proteins included proteins recognized by the αv and $\beta 1$ polyclonal antibodies (Fig. 5). Reactivity with the $\beta 1$ antibody differed between preparations of affinity-isolated oocyte plasma membrane: one preparation reacted strongly whereas a second reacted weakly (only slightly above the control reaction). Analysis of $\beta 1$ antiserum binding was repeated on these preparations and the same results were observed. The reactivity with the αv antibody was consistently strong.

Western sandwich blots

The $\beta 1$ antibody bound to two bands (137 and 93 kDa) when sperm plasma membrane proteins were first incubated with oocyte plasma membrane (Fig. 6); the $\beta 1$ antibody did not bind to sperm plasma membrane proteins in the absence of oocyte plasma membrane. The αv antibody did not bind specifically to the sperm plasma membrane protein blot pre-incubated with oocyte plasma membrane. Antibodies to α integrin subunits did not bind to these sperm plasma

Fig. 4. Western blot of pig oocyte plasma membrane proteins. Lane 1 was probed with normal rabbit serum and lane 2 was probed with the αv integrin subunit antibody. The M_r standards are indicated on the left-hand side.

Fig. 5. Slot blots of pig oocyte plasma membrane proteins eluted from a sperm plasma membrane affinity column. Slots were incubated with normal rabbit serum (NRS), $\beta 1$ or αv integrin subunit antibodies as indicated.

Fig. 6. Sandwich blots of pig sperm plasma membrane proteins. Integrin subunits were detected with specific anti-integrin subunit antibodies followed by a peroxidase-labelled second antibody. (a) Sperm plasma membrane proteins blocked once before incubation with the oocyte plasma membrane preparation. Lanes 1, 2 and 3 were incubated with oocyte plasma membrane proteins followed by αv or $\beta 1$ antiserum, or normal rabbit serum, respectively. Lanes 4 and 5 were incubated with the $\beta 1$ antiserum or normal rabbit serum, respectively (no oocyte plasma membrane proteins). Arrowheads indicate 137 and 93 kDa sperm proteins (and a minor band at 65 kDa) in lane 2 binding $\beta 1$ integrin subunits in the oocyte plasma membrane. (b) Sperm plasma membrane proteins blocked before and after incubation with the oocyte plasma membrane proteins. Lane 1 shows the 137 and 93 kDa sperm plasma membrane proteins binding $\beta 1$ integrin subunits in the oocyte plasma membrane. Lanes 2, 3, 4, 5 and 6 illustrate the absence of binding of normal rabbit serum and antiserum specific to $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 5$ integrin subunits to the sperm plasma membrane protein. The M_r standards are indicated on the right-hand side.

membrane protein blots, indicating that the 137 and 93 kDa sperm plasma membrane proteins were ligands for the $\beta 1$ integrin subunit in the oocyte plasma membrane rather than α subunits interacting with a β subunit.

Inhibition of sperm–oocyte interaction

Pig sperm–oocyte binding was reduced by the $\beta 1$ antibody (1:300 dilution) compared with the control antibody ($\alpha 5$; Fig. 7). Population distributions of spermatozoa binding to oocytes

Fig. 7. Inhibition of sperm-oocyte plasma membrane interaction by antibody to the $\beta 1$ integrin subunit. (a) Reduction in the number of spermatozoa bound to the oocyte plasma membrane. Bars represent means from three replicates with standard error bars (total of 181 oocytes examined). (b) Population distribution of oocytes by number of spermatozoa bound. Data from the two treatments in a single replicate are represented: $\alpha 5$ control (---) and $\beta 1$ (—).

in each replicate demonstrated a reduction of sperm binding to all oocytes in the presence of the $\beta 1$ antibody rather than a large change at one end of the distribution. In a preliminary experiment, there was no difference in sperm binding in the presence of a 1:300 dilution of control antiserum ($\alpha 5$) or in the absence of serum (102 versus 117). The reduction in sperm binding was not sufficient to reduce fertilization rates (62% versus 63% for $\beta 1$ and the control $\alpha 5$ antiserum, respectively, SEM = 3) or spermatozoa that penetrated per oocyte (2.8 versus 2.3, SEM = 0.8).

Discussion

The binding of polyclonal antibodies to αv and $\beta 1$ integrin subunits to the surface of pig oocytes indicates that these integrin subunits are part of the pig oocyte plasma membrane. Binding of a polyclonal antibody that recognizes the extracellular portion of the $\beta 1$ integrin subunit to unfixed oocytes supports the contention that this subunit is exposed on the surface of the pig oocyte. The available polyclonal antibody to the αv integrin subunit was to the cytoplasmic

tail of the molecule, hence an exposed surface localization of the αv subunit could not be verified. Identification of proteins by these integrin subunit antibodies on western blots is further evidence that these two integrin subunits are part of the oocyte plasma membrane. Although the estimated M_r values of oocyte plasma membrane proteins recognized by these two integrin subunit antibodies are only approximations from mini-gels, the values are in the appropriate range for integrin subunits (Hemler, 1990). Recognition of three distinct bands by the $\beta 1$ antibody was unexpected, but a similar result was observed in testicular tissue (Giebel *et al.*, 1997).

In contrast to murine oocytes (Almeida *et al.*, 1995), the $\alpha 6$ subunit did not appear to be a prominent component of the pig oocyte plasma membrane since no labelling was observed. The very weak reactivity observed in a small fraction of oocytes incubated with the $\alpha 3$ and $\alpha 4$ antibodies and the absence of labelling with the $\alpha 1$, $\alpha 2$ and $\alpha 5$ antibodies indicates that these subunits are not present or are present in very small numbers on the surface of the pig oocyte. The presence of very small numbers of some integrin subunits on rodent and human oocytes is implied by discrepancies among studies in the ability to detect specific subunits, in conjunction with differences in the sensitivity of different detection systems (Campbell *et al.*, 1995; Evans *et al.*, 1995; de Nadai *et al.*, 1996; Capmany *et al.*, 1998).

The inhibition of sperm-oocyte plasma membrane binding by an antibody recognizing an extracellular portion of the $\beta 1$ integrin subunit implies involvement of the $\beta 1$ moiety in sperm-oocyte interaction. The small but significant reduction in binding is consistent with $\beta 1$ integrin having an ancillary rather than an essential role in sperm-oocyte interactions. However, there are alternative explanations for the antibody inhibition of sperm-oocyte binding, such as an effect of antibody binding on membrane fluidity since even monovalent antibodies can occasionally affect membrane fluidity (Chasis and Mohandas, 1992). These trials were done with a divalent antibody since fragments with antigen binding (FABs) were not readily available. The presence of the $\beta 1$ integrin subunit in the oocyte plasma membrane proteins eluted from the sperm plasma membrane affinity column also indicates that the $\beta 1$ integrin subunit may be a receptor for a sperm plasma membrane protein. Binding of the $\beta 1$ integrin antibody to oocyte plasma membrane bound to sperm plasma membrane proteins with estimated M_r values of 137 and 93 kDa on a sandwich blot also supports a role for a $\beta 1$ integrin subunit in sperm-oocyte interaction. This observation indicates that these two sperm plasma membrane proteins of high M_r are the sperm ligands for the oocyte $\beta 1$ integrin subunit. Neither sperm plasma membrane protein band bound any of the polyclonal antibodies to seven common α integrin subunits, indicating that the interactions between the $\beta 1$ integrin unit in the oocyte plasma membrane and the sperm plasma membrane proteins were ligand-receptor interactions rather than associations of integrin subunits. Although both the αv and the $\beta 1$ integrin subunits could be detected in the oocyte plasma membrane fraction bound to the sperm plasma membrane affinity column, only the $\beta 1$ subunit appeared to

bind to a sperm plasma membrane in the sandwich blot. The binding of a β integrin subunit to a sperm plasma membrane protein is consistent with the study of Evans *et al.* (1997), in which the $\beta 1$ integrin subunit appeared to bind to the sperm fertilin β in mice. However, the α integrin subunit has also been proposed as the binding molecule for fertilin β (Almeida *et al.*, 1995; Chen and Sampson, 1999; Chen *et al.*, 1999).

The involvement of integrins in sperm–oocyte interaction was initially supported by the presumed essential role of the complementary molecule fertilin. The observation that spermatozoa from mice lacking fertilin have some ability to fuse with the oocyte plasma membrane (Cho *et al.*, 1998) indicates a facilitatory rather than an essential role for fertilin and its complementary receptor (Fenichel and Durand-Clement, 1998). However, the presence of cyritestin provides another ADAM family member to function as an integrin ligand in mouse sperm adhesion, and other less well characterized ADAM family members might also function in this role (Wolfsberg *et al.*, 1995b; Yuan *et al.*, 1997). The presence of fertilin α and cyritestin as pseudogenes in primates may be compensated by the presence of other related proteins that are complementary to integrin receptors (Jury *et al.*, 1997; Frayne and Hall, 1998; van Huijsduijnen, 1998). However, Ji *et al.* (1998) suggested that the $\beta 1$ integrin subunit is a facilitatory rather than an essential receptor in human gamete interaction.

The M_r values of the sperm plasma membrane proteins binding the $\beta 1$ oocyte integrin subunit are higher than would be expected for fertilin; the bovine sperm fertilin heterodimer is approximately 80 kDa under similar electrophoretic conditions (Waters and White, 1997). Alternatively, osteopontin is known to bind to the $\alpha v \beta 1$ integrin (Hu *et al.*, 1995). Osteopontin is a component of bovine seminal plasma and is located on rat sperm plasma membrane (Siiteri *et al.*, 1995), but there is no evidence to indicate that it is present on the plasma membrane in pig spermatozoa.

It is unclear whether an interaction with an integrin receptor is a major contributor to sperm–oocyte plasma membrane interaction of intact pig gametes. In the initial western ligand blots, sperm plasma membrane proteins with similar M_r values to those binding the oocyte $\beta 1$ integrin subunit appeared as minor rather than predominant binders of the biotinylated oocyte plasma membrane, and the extent of binding was not greater than that demonstrated in the most prominent non-tissue-specific proteins (Ash *et al.*, 1995). It is possible that this amount of binding on western ligand blots is unimportant, or alternatively the extent of binding may have been reduced compared with other proteins due to a greater reduction in biological activity during the harsh conditions of gel electrophoresis and blotting (Ocrant *et al.*, 1992).

It is likely that some, but not all, of the receptor–ligand interactions involved in sperm–oocyte interaction are tissue specific and specific to mammalian orders. Differences among species in integrin subunits may contribute to species-specific binding. However, the widespread presence of integrin molecules on cell surfaces contributes to the question of tissue specificity. Alternative splicing as proposed for the $\alpha 6$ and $\beta 1$ integrin subunits might provide a tissue-specific mechanism for involvement (Altruda *et al.*, 1990; Zucotti *et al.*,

1998). An oocyte-specific sequence would support the hypothesis that an integrin was involved in tissue-specific sperm–oocyte interaction. Polyclonal antibodies, as were used in the present study (and many monoclonal antibodies), would be expected to recognize the similarities in the sequences rather than detect potentially limited but functionally important differences. Alternatively, an integrin moiety may be involved in sperm–oocyte interaction after a tissue-specific step, which might modify the ligand or receptor. Interaction of an integrin with another oocyte molecule in a gamete-specific manner might also modify binding affinity.

The results of the present study indicate that the $\beta 1$ and αv integrin subunits are present in the plasma membrane in pig oocytes and that at least the $\beta 1$ subunit is exposed on the surface of the oocyte. These subunits showed an affinity for sperm plasma membrane proteins and the $\beta 1$ subunit recognized two sperm plasma membrane proteins of 137 and 93 kDa. Antibody binding to the extracellular portion of the $\beta 1$ subunit inhibited sperm–oocyte plasma membrane binding. Although the role of the $\beta 1$ integrin subunit on the oocyte plasma membrane remains to be determined, these observations are consistent with a role for $\beta 1$ integrin in sperm–oocyte interaction in pigs, possibly as a facilitating rather than an essential molecule.

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References

- Almeida EAC, Huovila APJ, Sutherland AE *et al.* (1995) Mouse egg integrin $\alpha_6 \beta_1$ functions as a sperm receptor *Cell* **81** 1095–1104
- Altruda F, Cervella P, Tarone G, Botta C, Balzac F, Stefanuto G and Silengo L (1990) A human integrin beta 1 subunit with a unique cytoplasmic domain generated by alternative mRNA processing *Gene* **95** 261–266
- Ash KL, Berger T, Horner CM and Famula TR (1994a) Boar sperm plasma membrane protein profile – correlation with the zona-free hamster ova bioassay *Theriogenology* **42** 1217–1226
- Ash K, Berger T, Munn RJ and Horner CM (1994b) Isolation and partial purification of plasma membrane from porcine oocytes *Molecular Reproduction and Development* **38** 334–337
- Ash K, Berger T, Horner CM and Calvert CC (1995) Identification of porcine sperm plasma membrane proteins that may play a role in sperm–egg fusion *Zygote* **3** 163–170
- Berger T and Horton MB (1988) Evaluation of assay conditions for the zona-free hamster ova bioassay of boar sperm fertility *Gamete Research* **19** 101–111
- Blobel CP, Wolfsberg TG, Turck CW, Myles DG, Primakoff P and White JM (1992) A potential fusion peptide and integrin ligand domain in a protein in sperm–egg fusion *Nature* **356** 248–252
- Campbell S, Swann HR, Seif MW, Kimber SJ and Aplin JD (1995) Cell adhesion molecules on the oocyte and preimplantation human embryo *Human Reproduction* **10** 1571–1578
- Capmany G, Mart M, Santalo J and Bolton VM (1998) Distribution of alpha (3), alpha (5) and alpha (v) integrin subunits in mature and immature human oocytes *Molecular Human Reproduction* **4** 951–956
- Chasis JA and Mohandas N (1992) Red blood cell glycoproteins *Blood* **80** 1869–1879
- Chen MS and Sampson NS (1999) Mediation of sperm–egg fusion: evidence that mouse egg $\alpha_6 \beta_1$ integrin is the receptor for sperm fertilin β *Chemistry and Biology* **6** 1–10
- Chen MS, Almeida EAC, Huovila APJ, Takahashi Y, Shaw LM, Mercurio AM and White JM (1999) Evidence that distinct states of the integrin $\alpha 6 \beta 1$ interact with laminin and an ADAM *Journal of Cell Biology* **144** 549–561

- Cho C, Bunch DO, Faure J, Goulding EH, Eddy EM, Primakoff P and Myles DG (1998) Fertilization defects in sperm from mice lacking fertilin β *Science* **281** 1857–1859
- de Nadai C, Fenichel P, Donzeau M, Epel D and Ciapa B (1996) Characterisation and role of integrins during gametic interaction and egg activation *Zygote* **4** 31–40
- Evans JP (1999) Sperm disintegrins, egg integrins, and other cell adhesion molecules of mammalian gamete plasma membrane interactions *Frontiers in Bioscience* **4** 114–131
- Evans JP, Schultz RM and Kopf GS (1995) Identification and localization of integrin subunits in oocytes and eggs of the mouse *Molecular Reproduction and Development* **40** 211–220
- Evans JP, Schultz RM and Kopf GS (1997) Characterization of the binding of recombinant mouse sperm fertilin α subunit to mouse eggs: evidence for function as a cell adhesion molecule in sperm–egg binding *Developmental Biology* **187** 94–106
- Fenichel P and Durand-Clement M (1998) Role of integrins during fertilization in mammals *Human Reproduction* **13** Supplement **4** 31–46
- Frayne J and Hall L (1998) The gene for the human tMDC I sperm surface protein is non-functional: implications for its proposed role in mammalian sperm–egg recognition *Biochemical Journal* **334** 171–176
- Giebel J, Loster K and Rune GM (1997) Localization of integrin $\beta 1$, $\alpha 1$, $\alpha 5$ and $\alpha 9$ subunits in the rat testis *International Journal of Andrology* **20** 3–9
- Hardy CM and Holland MK (1996) Cloning and expression of recombinant rabbit fertilin *Molecular Reproduction and Development* **45** 107–116
- Hemler ME (1990) VLA proteins in the integrin family: structures, functions, and their role on leukocytes *Annual Review of Immunology* **8** 365–400
- Hu DD, Lin ECK, Kovach NL, Hoyer JR and Smith JW (1995) A biochemical characterization of the binding of osteopontin to integrins $\alpha \beta_1$ and $\alpha \beta_5$ *Journal of Biological Chemistry* **270** 26 232–26 238
- Ji YZ, Wolf JP, Jouannet P and Bomsel M (1998) Human gamete fusion can bypass $\beta 1$ integrin requirement *Human Reproduction* **13** 682–689
- Jury JA, Frayne J and Hall L (1997) The human fertilin α gene is non-functional: implications for its proposed role in fertilization *Biochemical Journal* **321** 577–581
- Koehler JK, DeCurtis I, Stenchever MA and Smith C (1982) Interaction of human sperm with zona-free hamster eggs: a freeze-fracture study *Gamete Research* **6** 371–386
- McLaughlin EA, Frayne J, Barker HL, Jury JA, Jones R, Ford WCL and Hall L (1997) Cloning and sequence analysis of rat fertilin α and β – developmental expression, processing and immunolocalization *Molecular Human Reproduction* **3** 801–809
- Myles DG, Kimmel LH, Blobel CP, White JM and Primakoff P (1994) Identification of a binding site in the disintegrin domain of fertilin required for sperm–egg fusion *Proceedings National Academy of Science USA* **91** 4195–4198
- Ocran I, Fay C, Pham H and Rosenfeld R (1992) Not all insulin-like growth factor binding proteins (IGFBPs) are detectable by western ligand blotting: case studies of PC12 pheochromocytoma and rat anterior pituitary IGFBPs and proteolysed IGFBP-3 *Endocrinology* **131** 221–227
- Peterson R, Russell L, Bundman D and Freund M (1980) Evaluation of the purity of boar sperm plasma membranes prepared by nitrogen cavitation *Biology of Reproduction* **23** 637–645
- Schagger H and von Jagow G (1987) Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa *Analytical Biochemistry* **166** 368–379
- Siiteri JE, Ensrud KM, Moore A and Hamilton DW (1995) Identification of osteopontin (OPN) mRNA and protein in the rat testis and epididymis, and on sperm *Molecular Reproduction and Development* **40** 16–28
- Talbot P and Chacon RS (1982) Ultrastructural observations on binding and membrane fusion between human sperm and zona pellucida-free hamster oocytes *Fertility and Sterility* **37** 240–248
- Tarone G, Russo MA, Hirsch E, Odorisio T, Altruda F, Silengo L and Siracusa G (1993) Expression of $\beta 1$ integrin complexes on the surface of unfertilized mouse oocytes *Development* **117** 1369–1375
- van Huijsduijnen RH (1998) ADAM 20 and 21; two novel human testis-specific membrane metalloproteases with similarity to fertilin α *Gene* **206** 273–282
- Waters SI and White JM (1997) Biochemical and molecular characterization of bovine fertilin alpha and beta (ADAM 1 and ADAM 2): a candidate sperm–egg binding/fusion complex *Biology of Reproduction* **56** 1245–1254
- Wheeler E, Berger T and Behboodi E (1996) Bovine oocyte plasma membrane binding sites for sperm plasma membrane during *in vitro* oocyte maturation and fertilization *Zygote* **4** 67–72
- Wolfsberg TG, Primakoff P, Myles DG and White JM (1995a) ADAM, a novel family of membrane proteins containing a disintegrin and metalloprotease domain: multipotential functions in cell–cell and cell–matrix interactions *Journal of Cell Biology* **131** 275–278
- Wolfsberg TG, Straight PD, Gerena RL, Huovila AJ, Primakoff P, Myles DG and White JM (1995b) ADAM, a widely distributed and developmentally regulated gene family encoding membrane proteins with a disintegrin and metalloprotease domain *Developmental Biology* **169** 378–383
- Yanagimachi R (1994) Mammalian fertilization. In *The Physiology of Reproduction* pp 189–317 Eds E Knobil and JD Neill. Raven Press, New York
- Yuan R, Primakoff P and Myles DG (1997) A role for the disintegrin domain of cyritestin, a sperm surface protein belonging to the ADAM family, in mouse sperm–egg plasma membrane adhesion and fusion *Journal of Cell Biology* **137** 105–112
- Zheng YS, Fiser P and Sirard MA (1992) The use of ejaculated boar semen after freezing in 2 or 6 percent glycerol for *in vitro* fertilization of porcine oocytes matured *in vitro*. *Theriogenology* **38** 1065–1075
- Zuccotti M, Rossi PG, Fiorillo E, Garagna S, Forabosco A and Redi CA (1998) Timing of gene expression and oolemma localization of mouse α_6 and β_1 integrin subunits during oogenesis *Developmental Biology* **200** 27–34