Original Article

MARINE BIOTECHNOLOGY

Apparent Involvement of a β1 Type Integrin in Coral Fertilization

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Abstract

Integrins are involved in a wide variety of cell adhesion processes, and have roles in gamete binding and fusion in mammals. Integrins have been also discovered in the scleractinian coral *Acropora millepora* (Cnidaria: Anthozoa). As a first step toward understanding the molecular basis of fertilization in corals, we examined the effect of polyclonal antisera raised against recombinant coral integrins on gamete interactions in *A. millepora*. Antiserum raised against integrin βcn1 dramatically decreased the binding of *Acropora* sperm to eggs and significantly decreased fertilization rates relative to preimmune serum and seawater controls. However, the antiserum against AmIntegrin α1 did not affect significantly either sperm–egg binding or fertilization. One possible explanation for this is that AmIntegrin α1 may preferentially mediate interactions with RGD-containing ligands, whereas mammalian α6 integrin (which is most directly implicated in gamete interactions) preferentially interacts with laminin-related ligands. Our results suggest that β1 type integrins are involved in the fertilization process in *Acropora* and that some functions of these molecules may have been conserved between corals and mammals.

Keywords: *Acropora millepora* — coral — fertilization — gamete interaction — integrin

Introduction

Many reef-building corals participate in synchronous mass spawning events (Babcock et al., 1986; Harrison et al., 1984; Hayashibara et al., 1993), which represents opportunities for hybridization among different species. Interspecific hybridization has been demonstrated in a number of coral genera, and appears to have been a major factor in the evolutionary success of *Acropora*, which dominates coral reefs in the Indo-Pacific and is one of the most widespread, abundant, and species rich (113 to 180 species) of coral genera (Wallace, 1999; Veron, 2000; Willis et al., 2006). However, the application of population genetic approaches (Márquez et al., 2002a, b) has demonstrated that while hybridization is important on evolutionary time scales, it occurs only rarely on ecological time scales even between species such as *A. hyacinthus* and *A. cytherea*, which are highly cross-fertile in in vitro trials (mean=50%) (Willis et al., 1997), occur in sympatry, and spawn synchronously. This fact implies that substantial, albeit incomplete, prezygotic barriers exist to hybridization between coral species, although the nature of these isolating mechanisms and the molecular bases of gamete interactions remain unknown.

Mammalian fertilization has been the subject of intense investigation, and a number of proteins mediating sperm-egg binding and membrane fusion have been identified (Evans, 2002; Kaji and Kudo, 2004). In a classic series of experiments, Almeida et al. (1995) showed that integrin α6β1 is likely to play a critical role on the egg side in gamete interactions in the mouse, the likely ligands on the sperm side belonging to the ADAM (*a d*isintegrin *a*nd a *m*etalloprotease) family. Candidate ADAM molecules in this context are ADAM1 (fertilin α), ADAM2

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(fertilin β), and ADAM3 (cyritestin) (Evans, 2002); ADAM1, ADAM2, or ADAM3 fusion proteins can bind to the mouse egg plasma membrane and inhibit fertilization (Bigler et al., 2000; Eto et al., 2002; Evans et al., 1997a, b). Despite some discrepancies between results obtained using either antibodies or fusion proteins and those based on knockout mice (Miller et al., 2000; He et al., 2003), the evidence supporting roles for ADAM–integrin interactions during sperm–egg binding and membrane fusion is compelling (Almeida et al., 1995; Ziyyat et al., 2006).

As a first approach to unraveling the molecular bases of gamete interactions in cnidarians, we examined the ability of antibodies raised against homologous integrins to inhibit sperm-egg binding and fertilization. These experiments essentially replicate those of Almeida et al. (1995) on the mouse system; justifications for extrapolating across such a great evolutionary distance include the remarkable similarity between the anthozoan and vertebrate gene repertoires (Kortschak et al., 2003; Technau et al., 2005) and striking parallels in patterns of expression of some genes (reviewed in Ball et al., 2004). In these experiments, we focused on two integrin molecules (integrin βcn1, AmIntegrin α1) that have previously been identified in the coral *Acropora millepora* (Brower et al., 1997; Knack et al., submitted). Integrins function as α/β heterodimers; higher animals typically have several α subunits (>10 in mammals) capable of associating with β1 integrin subunits (Hynes, 2002). Coral integrin βcn1 is similar to the mammalian β1 type, which has been implicated in the fertilization process (see above) and, although not directly orthologous, AmIntegrin α1 is most similar at the primary sequence level to the vertebrate α4/9 type integrins (Knack et al., submitted).

Materials and Methods

Expression Analysis by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Unfertilized eggs were collected from a single colony of *Acropora millepora*. Total RNA of unfertilized eggs was isolated using RNeasy Mini Kit (QIAGEN, Chatsworth, CA) according to the manufacturer's protocol. RNA was visualized on 1% agarose gel with ethidium bromide under ultraviolet light. RNA was treated with DNase (Fermentas, Glen Burnie, MD) to remove contaminating genomic DNA. Single-stranded cDNA (sscDNA) was synthesized using First-strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ) with above 1 μg of total RNA. One microliter of the sscDNA was used per 20 μl of the PCR. For integrin βcn1 (Brower et al., 1997), a primer pair IB1RTF (5′-CTTGTGTTGCCACTTAT GGCTT-3′) and IB1RTR (5′-CTGCTACTTGCA TTAACGCATC-3′) amplifying a fragment of 144 bp was used. For AmIntegrin α1, a primer pair AmItga1RTF (5′-GCCAATGAAACAGC-TACG-3′) and AmItga1RTR (5′-TTGTCTCCAGCCTT-CAAC-3′) amplifying a fragment of 130 bp was used. The PCR profile was as follows: 1 min at 94°C, 40 cycles of 0.5 min at 94°C (denaturation), 0.5 min at 50°C (annealing), 2 min at 72°C (extension), followed by an additional extension for 2 min at 72°C. The PCR products were visualized on 1% agarose gel with ethidium bromide under ultraviolet light. As a positive control for fidelity and efficiency of the amplification, partial cDNA of coral actin was amplified by PCR with ActinF (5′-CGAACACGGA ATCGTAACCAACTGG-3′) and ActinR (5′-CAAA TCCAGACGTAAGATGG-CATGG-3′). These primers were designed based on the sequence of Actin EST clone (unpublished data).

Expression of Recombinant Integrin Proteins. A DNA fragment encoding the N-terminal 155 amino acids of integrin βcn1 (AmItgb1) which includes DxSxS motif in the MIDAS domain) was generated by PCR from the integrin βcn1 cDNA plasmid with a 5′ primer engineered with a *Bam*HI restriction site (5′-CGCGGATCCGCGATGAAGCGAAGGCTT TGCTTG-3′) and a 3′ primer with a *Hae*III site (5′-GGGGCCCCCTTGTCCACAAATGAACCAAA ACCAAG-3′). This fragment was inserted into *Bam*HI and *Hae*III restriction sites of the pQE30 vector (QIAGEN). A DNA fragment of 144 amino acids of the head region beta-propeller repeats 5, 6, and 7, including the three DxD/NxD/NxxxD motifs of AmIntegrin α1 (AmItga1) was generated via PCR from the AmIntegrin α1 cDNA plasmid with a 5′ primer engineered with a *Bam*HI restriction site (5′-GGATCCAGTGTTGTTTGTGCTGTG-3′) and a 3′ primer with a *Xho*I site (5′-CTCGAGCATACG CGCCGACAACAAT-3′). This fragment was inserted into *Bam*HI and *Xho*I restriction sites of the pGEX-4T-2 vector (Amersham). Restriction enzyme digestion, ligation, and transformation were performed according to standard protocols (Sambrook et al., 1992).

Integrin βcn1 and AmIntegrin α1 fusion proteins were expressed in *Escherichia coli* XL1 Blue cells. The cultures were grown at 37°C to an OD_{600} of 0.4 before inducing by adding isopropyl-β-d-thiogalacto-pyranoside (IPTG) to a final concentration of 1 mM and then incubated for a further 4 h. The cells were then lysed by sonication and separated into soluble and insoluble fractions by centrifugation. Both fusion proteins were recovered in membrane (rather

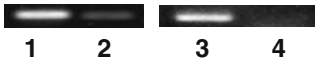

Figure 1. Expression of integrin βcn1 *(lane 1)* and AmIntegrin α1 *(lane 2)* in unfertilized egg of *Acropora millepora*. *Lane 3* corresponds to the positive control (actin), and lane 4 corresponds to the negative (no template) control.

than soluble) fractions and could not be purified using an affinity chromatography. Therefore, the integrin βcn1fusion protein was purified by preparative electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide (15%) gels followed by electroelution. The fusion protein of AmIntegrin α1was purified by washing the insoluble fraction with 50 mM Tris-HCl (pH 9.0) including 2% Triton X-100.

Antibody Production. For antibody production, rabbits were immunized using four aliquots of the purified protein (300 to 400 μg/dose) in Freund's complete (first injection) or incomplete (all other injections) adjuvant. Injections were given at intervals of 2 weeks. One week after the final injection, an aliquot of serum was evaluated for antibody titer by Western analysis, and the rabbits were bled out within 24 h of this. The blood was allowed to clot at room temperature for 3 h and stored overnight at 4°C to allow the clot to contract, after which the clot was manually removed and the serum was collected. Antibody production was performed by IMVS Veterinary Services (South Australia). The sensitivity and specificity of the antibodies were confirmed by their use to probe Western blots of whole *E. coli* lysates with or without the recombinant antigen, and signals were detected as a single band.

Fertilization Trials. Gravid coral colonies were collected from fringing reefs in Geoffrey and Nelly Bay at Magnetic Island, off Townsville (Queensland, Australia), and from NE Reef on Orpheus Island of the Great Barrier Reef. Gametes were collected and prepared following Willis et al. (1997). The reciprocal of each sperm–egg combination was considered to be a separate cross. All crosses were performed in 1 ml volumes in 3 ml vials and replicated three times. Between 10 and 100 eggs were incubated at room temperature for at least 15 minutes with each treatment (seawater, antiserum, antibody-free serum) before the sperm was added. Optimal concentration of 10^6 sperm/ml (Willis et al., 1997) was used for each cross. Eggs were fixed with formalin-β-glycerophosphate 6 h after addition of sperm, and the number of unfertilized eggs and developing embryos was counted under a dissecting microscope.

To determine whether the antisera inhibit the binding of sperm to eggs, phase-contrast microscopy was carried out on *A. millepora* eggs that had been fixed 45 min after addition of sperm. Eggs were washed with filtered seawater to remove unbound sperm prior to slide preparation.

Results and Discussion

As can be seen from RT-PCR experiments (Figure 1), mRNAs encoding integrin βcn1 and AmIntegrin α1 are present in unfertilized *Acropora* eggs. Maternal integrin mRNAs have also been reported in a wide variety of other animals (e.g., Whittaker and DeSimone, 1993), and in the hydrozoan *Podocoryne*, a homolog of integrin βcn1 and a putative ortholog of AmIntegrin α1 are likewise maternal (Reber-Muller et al., 2001).

Phase-contrast microscopy revealed that the antiserum against integrin βcn1 dramatically decreased the binding of conspecific sperm to *Acropora* eggs (Figure 2) and significantly decreased fertilization rates (Figure 3). In Figure 2A and 2B, large numbers of sperm can be seen bound to eggs that had been preincubated with the antiserum control (preimmune serum). In contrast, none or very few sperm were bound to eggs that had been preincubated with the antiserum against integrin βcn1 (Figure 2C and 2D). Fertilization rates were reduced when *A. millepora* eggs were pretreated with antiserum (0.5 dilution in 2× artificial seawater) developed against integrin βcn1 (Figure 3). Mean fertilization success was reduced by approximately 20% (to an average of 68.7±4.16%) compared to the seawater (87.4±2.38%) (paired *t*-test, $P<0.01$) and serum controls (83.4±3.19%) (paired *t*-test, $P<0.05$).

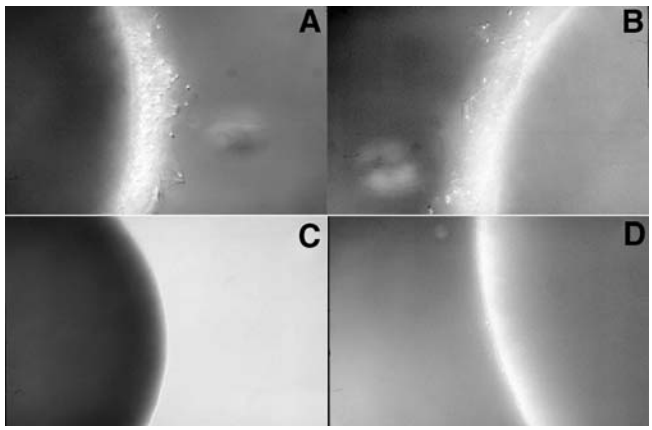

Figure 2. Phase-contrast microscopic images of untreated *Acropora millepora* eggs ***(A, B)*** or treated with antiserum against AmItgb1-His for 15 min ***(C, D)*** before addition of 10^6 sperm/ml.

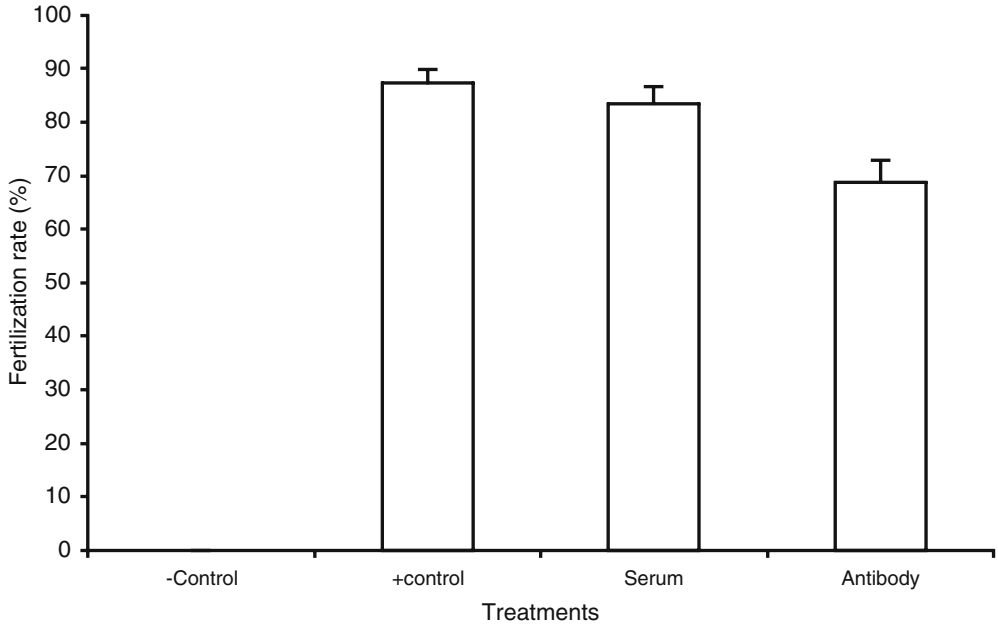

Figure 3. Fertilization rates (%) of *Acropora millepora* eggs treated with antiserum against AmItgb1-His protein. Bars show average fertilization rates and standard errors for 11 crosses (n=37). Each treatment was repeated three times per cross. Sperm were added at a concentration of 10^6 sperm/ml. Control: only eggs and no sperm in 1× artificial seawater; + Control: eggs and sperm in 1× artificial seawater; Serum: eggs and sperm in 0.5 dilution of rabbit serum in 1× artificial seawater; Antibody: eggs and sperm in 0.5 dilution of rabbit serum containing antibody against *A. millepora* AmItgb1-His protein in 1× artificial seawater.

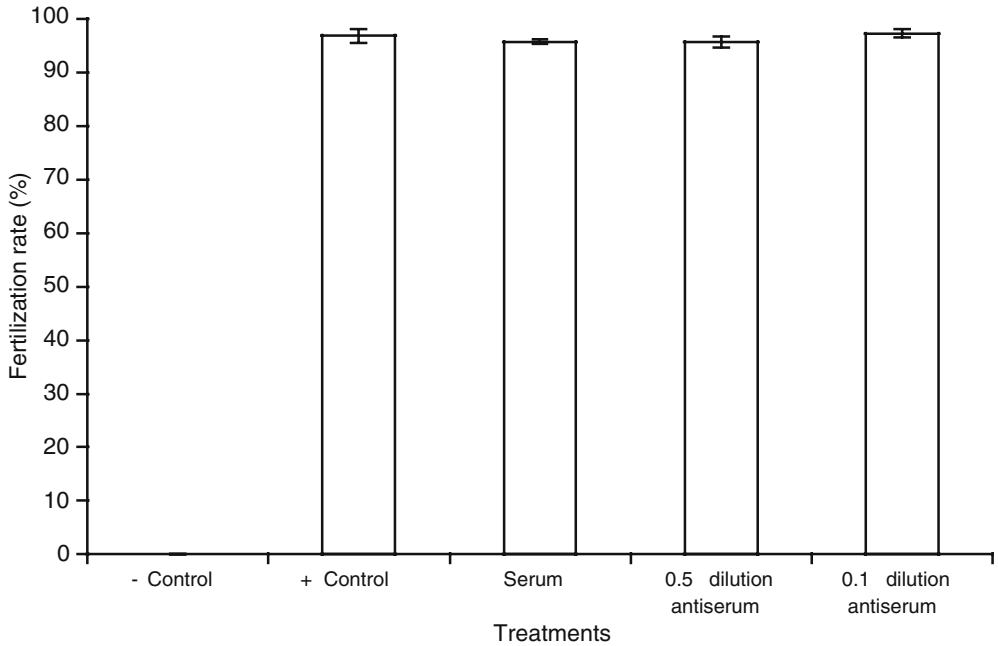

Figure 4. Fertilization rates (%) of *Acropora millepora* eggs treated with different doses of antiserum against AmItga1-GST protein. Bars show average fertilization rates and standard errors for three crosses (n=9). Each treatment was repeated three times per cross. Sperm were added at a concentration of 10^6 sperm/ml. Treatment descriptions as in Figure 3, with the exception of the dilution of the serum with antibody.

The apparent inconsistency between the effects of anti-integrin βcn1 on sperm binding and fertilization results from the very different levels of sensitivity of the two measures of inhibition—a decrease in sperm binding of 99.99% may still allow fertilization to occur because sperm are active even 6 h after spawning (Willis et al., 1997; Omori et al., 2001).

Whereas anti-integrin βcn1 clearly affected sperm–egg interactions, the antibody against AmIntegrin α1 did not appear to have any significant effects on either sperm binding or fertilization rates under the same conditions. Results from sperm binding experiments in the presence of antibody were indistinguishable from preimmune serum controls, and fertilization rates in the presence of anti-AmIntegrin α1 were 95.7 ± 0.99% compared with seawater (96.8±1.36%; Mann-Whitney, $P>0.05$) and pre-immune serum controls (95.8±0.46%; Mann-Whitney, $P>0.05$) (Figure 4). Sperm motility was normal under all antiserum treatments (data not shown).

This study showed that antibodies against integrin βcn1 protein of the coral *Acropora millepora* are able to partially inhibit fertilization in this species while those developed against AmIntegrin α1 did not affect fertilization. Although the possibility that the latter antibody recognizes epitopes that are inaccessible prior to gamete interaction (thus being unable to block the binding site) cannot yet be ruled out, these experiments suggest that, despite both integrins being maternally expressed, AmIntegrin α1 may not be involved in coral fertilization. Integrin β1-type molecules have been shown to dimerize with a number of different α subunits (Hynes, 2002), so it appears likely that additional α integrins are present in *Acropora*. Consistent with this, a preliminary investigation of the integrin complement of a related anthozoan, the sea anemone, *Nematostella vectensis* (for which a whole genome sequence is now available), suggests the presence of at least three distinct α integrin subunits (Knack et al., submitted).

The α subunit of the integrin heterodimer directly implicated in mammalian fertilization is α6 (Almeida et al., 1995), which confers specificity for laminin as ligand (Hynes, 2002). Although AmIntegrin α1 is most similar in sequence to the mammalian α4/9 type, its ligand binding properties remain unresolved (Knack et al., submitted). Among the suggested coral integrin complements are members which bind each laminin and RGD peptides preferentially. Priority should be placed on characterizing the ligand binding properties of the full complement of *Acropora* integrin alpha subunits and assessing their potential roles in gamete interactions.

Both the inhibition of sperm binding, demonstrated microscopically, and the reduction in fertilization rates of antiserum-treated eggs support our conclusion that integrin βcn1 plays a significant role in coral fertilization. Given the evolutionary position of corals—members of the basal class (the Anthozoa) within the "sister group" of the Bilateria (Ball et al., 2004), these findings suggest that integrins may play central roles in gamete interactions throughout the higher Metazoa.

The next challenge is to understand the roles of proteins making molecular complexes with integrin βcn1 in gamete interactions of *A. millepora*. In mammals, tetraspanins are also central players in gamete interactions, acting as receptors or co-receptors for the integrin ligand (Le Naour et al., 2000; Miyado et al., 2000). The association of integrins with various tetraspanins has also been often documented (Boucheix and Rubinstein, 2001). In an ongoing expressed sequence tag (EST) project on *Acropora millepora* (Kortschak et al., 2003), several tetraspanins have already been identified (unpublished data). Investigating the interactions of the *Acropora* tetraspanins with integrins and their potential involvement in gamete interactions is a priority to understand the complexities of coral reproduction and the role of these proteins through metazoan evolution.

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