Sequence-specific Interaction between the Disintegrin Domain of Mouse ADAM 2 (Fertilin β) and Murine Eggs

ROLE OF THE α6 INTEGRIN SUBUNIT*

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Little is yet known about the biochemical and biological properties of the disintegrin-like domains of ADAM (a disintegrin and metalloprotease) proteins. Mouse ADAM 2 (mADAM 2; fertilin β) is a sperm surface protein involved in murine fertilization. We produced recombinant proteins containing the disintegrin-like domain of mADAM 2 in both insect cells and in bacteria. The protein produced in insect cells (baculo D+C) contained a signal sequence followed by the disintegrin-like and cysteine-rich domains; it was purified from the medium of recombinant baculovirus-infected cells. A bacterial construct containing the disintegrin-like domain was produced in Escherichia coli as a glutathione S-transferase chimera. Baculo D+C, as well as the D domain of the bacterial construct (released with thrombin), bound to the microvillus surface of murine eggs. Using concentrations in the range of 1 to 5 μM, both recombinant proteins strongly inhibited sperm-egg binding and fusion; the baculovirus-produced protein exhibited a somewhat greater extent of inhibition (~75 versus ~55% maximal inhibition). Substitution of alanine for each of the five charged residues within the disintegrin loop of mADAM 2 revealed a critical importance for the aspartic acid at position nine. Binding of both recombinant proteins to the egg was inhibited by the function blocking anti-α6 monoclonal antibody, GoH3, but not by a nonfunction-blocking anti-α6 monoclonal antibody. Binding was also inhibited by a peptide analogue of, and with an antibody against, the disintegrin loop of mADAM 2.

ADAMs† are a large group of type I integral membrane glycoproteins that contain a disintegrin and a metalloprotease domain (1, 2). Following their metalloprotease and disintegrin-like domains, they contain a cysteine-rich domain, an EGF repeat, a transmembrane domain, and a cytoplasmic tail. Their closest relatives are the P-III snake venom metalloproteases (SVMPs), which are secreted proteins that contain a disintegrin-like and a metalloprotease domain as well as a cysteine-rich domain. P-II SVMPs contain metalloprotease and disintegrin domains but lack the cysteine-rich (and other) domains. The disintegrin domains of the P-II SVMPs have a 13-amino acid loop containing, at their tips, sequences such as RGD (kistrin and echistatin), KGD (barbourin), and MVD (atrolysin E). Several P-II snake disintegrins have been shown to bind to the platelet integrin, α1bβ3, thereby preventing binding of fibrinogen and inhibiting platelet aggregation. Inhibition of platelet aggregation accounts, in part, for the severe hemorrhagic response in snakebite victims. The disintegrin-like domains of P-III SVMPs differ from their P-II counterparts in having two additional cysteine residues and a 14-residue predicted loop that aligns with the 13-amino acid “RGD” loop found in the P-II disintegrins. One of the additional cysteine residues is near the center of the disintegrin loop.

The disintegrin-like domain of the P-III SVMP atrolysin A has been characterized. Atrolysin A purified from snake venom inhibits ADP- and collagen-induced platelet aggregation with an IC50 values of ~0.24 and ~0.11 μM, respectively. A recombinant protein produced in insect cells containing the disintegrin-like and cysteine-rich domains of atrolysin A inhibits ADP- and collagen-induced platelet aggregation with similar IC50 values of ~0.3 and ~0.47 μM, respectively. In contrast, a peptide analogue of the disintegrin loop of atrolysin A blocks collagen-induced platelet aggregation with an IC50 of ~300 μM.

Relatively little is known about the biochemical and biological properties of ADAM disintegrin-like domains. What is known can be summarized as follows: Peptide analogues of the disintegrin loops of several sperm ADAMs inhibit sperm-egg binding and fusion using peptide concentrations of 100–500 μM (4–13). Recombinant proteins encoding the extracellular domains of mature mouse ADAM (mADAM) 1 (fertilin α) and mature mADAM 2 (fertilin β), appended to the maltose-binding protein, have been expressed in Escherichia coli. When added to mouse eggs at a concentration of ~1 μM, the fertilin β monoclonal antibody; HA, hemagglutinin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; β-DCE, fertilin β extracellular domain.

† A "mature" ADAM subunit begins with its disintegrin-like domain. Developmentally mADAM 2 is processed to its mature form by sequential proteolytic removal of the pro and metalloprotease domains (14–17).
construct inhibited sperm-egg binding and fusion by ~50 and ~25%, respectively (18).

Human ADAM (hADAM) 15 is the only known ADAM that contains an RGD sequence in its disintegrin loop. A recombinant protein consisting of the disintegrin-like domain of hADAM 15, appended to glutathione S-transferase (GST) and expressed in E. coli, was shown to bind to cells expressing the α6β1 integrin but not to cells expressing other integrins including α5β1 and α3β1. Mutation of the RGD tripeptide to SGA blocked binding to α6β1 (19). A chimeric protein containing the entire extracellular domain of hADAM 15 attached to the IgG heavy chain and expressed in mammalian cells has been shown to bind to cells that express the α6β1 or α5β1 integrins. This binding was inhibited by an RGD peptide (20).

We previously provided evidence that the disintegrin-like domain of mADAM 2 interacts with the integrin α6β1: (i) Sperm binding to eggs is inhibited by a function-blocking anti-α6 monoclonal antibody (mAb) as well as by a peptide analogue of the disintegrin loop of mADAM 2. (ii) Sperm bind more avidly to α6-transfected cells than to their mock-transfected counterparts as well as to cells expressing versus those engineered not to express the β1 integrin subunit. (iii) Sperm binding to somatic cells expressing α6β1 is specifically inhibited by the function-blocking anti-α6 mAb GoH3, as well as a disintegrin loop peptide (4). Recent work from our laboratory, using mADAM 2 purified from sperm and captured onto fluorescent beads, is consistent with this conclusion: bead binding is inhibited by the anti-α6 mAb GoH3 and by a disintegrin loop peptide analogue (21). In addition, recent work has shown that a radioiodinated and photoactivatable peptide analogue of the disintegrin loop of mADAM 2 binds to the egg integrin α6β1 (6). However, results using a maltose-binding protein chimera of the entire extracellular domain of mature mADAM 2 produced in E. coli were interpreted as evidence that mADAM 2 interacts with a β1 integrin on the egg but not with α6β1 (22).

To gain a better appreciation for the biochemical and biological properties of the disintegrin-like domains of ADAM proteins and to test whether recombinant disintegrin domain-containing constructs of mADAM 2 can interact with the α6β1 integrin, we expressed proteins containing either the disintegrin-like domain or the disintegrin-like and cysteine-rich domains of mADAM 2 in both insect and bacterial cells. Recombinant proteins produced in both bacterial and insect cells bound to the egg surface and inhibited sperm-egg binding and fusion. A point mutant within the disintegrin loop (D9A) strongly reduced the ability of the recombinant mADAM 2 disintegrin domain to inhibit sperm-egg binding and fusion. In addition, binding of recombinant mADAM 2 disintegrin domains produced in both insect cells and in bacteria was inhibited by a function-blocking antibody against the α6 integrin subunit as well as by reagents that target the disintegrin loop of mADAM 2.

**EXPERIMENTAL PROCEDURES**

**Insect Cells—**Recombinant baculovirus infections were performed in High Five cells in serum-free medium (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. High Five cells were used for ~30 passages.

**Antibodies—**The rat anti-α6 integrin mAb GoH3 was obtained from Immunotech (Westbrook, ME). The rat anti-α6 integrin mAb J1B5 was obtained from Dr. Caroline Damsky (University of California, San Francisco, CA). An antibody against the disintegrin loop of mADAM 2 (fertilin β) was generated as follows: Five peptides overlapping the disintegrin loop sequence were synthesized and coupled, individually, by amide linkage to KLH. The peptides were: 1, KLRR-GEVCRLAQDE; 2, CRLAQDECDVTEY; 3, DVTYECNGTSEV; 4, CRLAQDECDVTEY; and 5, KLRRKGEVCRLAQDECDVTEYEN. ~100 μg of each peptide conjugate was mixed with Freund’s adjuvant and injected into the lymph nodes of rabbits. Rabbits were boosted once, and serum was collected and stored by standard procedures. For use, the antisemum was affinity-purified over a column of peptide 4. Fab fragments were generated using the ImmunoPure Fab Preparation Kit (Pierce) following the manufacturer’s instructions. Control Fab fragments were prepared from whole rabbit IgG molecules (Jackson, West Grove, PA) in the same way.

**Pepites—**14-mer peptides with the sequence from the disintegrin loop of mADAM 2 as well as a scrambled disintegrin loop sequence were synthesized on a Symphony multiple peptide synthesizer (Rainin) and purified by high pressure liquid chromatography. The sequences were: mADAM 2, CRLAQDECDVTEY; and scrambled mADAM 2, CRLAQDECDVTEYE. Peptides were amidated at the C terminus and acetylated at the N terminus. The two terminal cysteines were protected with acetoamidomethyl groups. Immediately before use, the peptides were dissolved in Me2SO to a concentration of 25 mM and diluted into egg medium.

**Gamete Isolation and Zona Pellucida Removal—**Eggs and sperm were obtained, respectively, from 8–10-week-old ICR female mice and ICR retired male breeders (Harlan, Indianapolis, IN). Mature oocytes were collected from female mice as described previously (4). Zonae pellucidae were removed by either the chymotrypsin-mechanical method as described by Almeida et al. (4) or by treatment with acidic Tyrode’s solution (pH 2.5) for 15–30 s as described in Chen et al. (21). The zona-free eggs were immediately washed three times with egg medium (20 min/wash) and allowed to recover for 1 h in the case of chymotrypsin-mechanical method treatment or 3 h in the case of treatment with acidic Tyrode’s solution. Sperm were isolated from the cauda epididymis of male mice as described previously (21) and capacitated for 2–3 h.

**Constructs Containing the Disintegrin-like and Cysteine-rich Domains (D+C) of mADAM 2—**A DNA fragment encoding the disintegrin-like and cysteine-rich domains of mADAM 2 was generated by polymerase chain reaction from a mouse fertilin β cDNA clone (23). The upstream primer was 5′-CGAGTCCAATCCTAGTATGCGCGTCCTGGG GA-3′ containing the N-terminal end of the disintegrin-like domain, corresponding to nucleotides 1162–1180 of GenBank™ Accession no. U16242 plus upstream BamHI and SnaBI restriction sites for in-frame insertion (using the BamHI site) into the baculovirus expression vector pVT-Bac (24), which contains the melittin signal sequence. In a second step, an hemagglutinin (HA) epitope tag was added at the C-terminal end using the following primer: 5′-CGGGATCCTAGGCATAATCTGG-9′. A chimeric protein containing the disintegrin-like domain of mature mADAM 2 produced in E. coli were interpreted as evidence that mADAM 2 interacts with a β1 integrin on the egg but not with α6β1 (22).

**Expression of Baculo D+C in Insect Cells—**Recombinant baculovirus encoding baculo D+C was generated using the BaculoGold system (Pharmingen, San Diego, CA) according to the manufacturer’s instructions. After three rounds of plaque purification, the stock virus was amplified and titered. The amplified virus stock was stored at 4 °C, covered with aluminum foil, for no longer than 3 months. The original virus stock was stored at ~80 °C in 500-μl aliquots for subsequent amplification as needed. For infection, High Five cells were split to a density of 1 × 10^6 cells/ml infected with virus at a multiplicity of infection of 2, and incubated for 72–96 h at 27 °C. Cells were pelleted, and the supernatant was filtered through an 0.2-μm sterile filter (Corning Plastics, Corning, NY).

**Purification of Baculo D+C from Insect Cell Medium—**Filtered medium from D+C encoding baculovirus-infected High Five cells was concentrated 10-fold through a Minicel concentrator (30,000 molecular weight cutoff; Amicon, Danvers, MA) and then purified on a column of TALON metal affinity resin (CLONTECH Laboratories, Palo Alto, CA). The resin was washed with binding buffer (20 mM Tris-HCl, pH 8.0; 100 mM NaCl; 10% glycerol) and the baculo D+C protein was bound at a ratio of 100 ml of concentrated medium/6 ml column resin by mixing at 4 °C overnight on a platform shaker. The TALON slurry with bound baculo D+C was poured into a column, and the column was then washed with binding buffer followed by washing buffer containing 1 mM imidazole. Baculo D+C was eluted in three column volumes of binding buffer containing 50 mM imidazole. Each elution was preceded by incubation for 15 min at 4 °C on an orbital shaker.

3 In the terminology of Fox and co-workers (3), our construct encodes the spacer, disintegrin-like, and cysteine-rich domains.
Disintegrin Domain of ADAM 2

platform shaker. The fractions were pooled and either dialyzed immediately or extensively against PBS using a Slide-A-Lyzer (10,000 molecular weight cutoff; Pierce) or immediately concentrated and washed three times with PBS in a centrifron concentrator (30,000 molecular weight cutoff; Amicon, Danvers, MA). The final concentration of baculovirus and baculovirus expression vector, were subcloned into the GST expression vector pGEX-4T-2 (Amersham Pharmacia Biotech). Synthesis of the GST-D fusion protein was induced in the DH5α strain of E. coli by adding 0.1 mM isopropyl-1-thio-β-galactopyranoside to a culture in log phase for 5–6 h at 37 °C. For expression of GST-D+C we used the BL21 strain of E. coli, a protease-deficient strain, because we observed significant degradation of the GST-D+C protein produced in DH5α cells.4 Synthesis of GST-D+C was induced by adding 0.1 mM isopropyl-1-thio-β-galactopyranoside for 12–16 h at 25 °C. The bacteria were pelleted, resuspended in cold PBS (1/20th of the original culture volume), and either passed twice through a French pressure cell for volumes greater than 10 ml (GST-D+C) or disrupted with a probe sonicator for smaller volumes (GST-D). The GST fusion proteins were then purified using glutathione-agarose (Sigma) affinity chromatography, according to the manufacturer’s instructions. For specified experiments, D was cleaved from GST-D by incubation with thrombin (Sigma); 1 unit of thrombin was added/100 μg of GST-D, and the solution was incubated overnight at 4 °C. The released D protein was then purified over a TALON spin column (CLONTECH Laboratories) according to the manufacturer’s instructions using the same binding, wash, and elution buffers described above for baculo D+C purification. The eluted fractions were dialyzed immediately and concentrated as above.

Treatment with N-Glycosidase F and Tunicamycin—Sf9 cells were infected with baculovirus encoding D+C for 24 h. Infected cells (~3 x 10^6) were then lysed, and the D+C protein precipitated with 50 μl of TALON beads for 2 h at 4 °C. Following three washes with TALON binding buffer (see above) the D+C protein was eluted by boiling for 3 min in N-glycosidase buffer (1% N-acytethylglucoside, 0.2% SDS, 40 mM Tris, pH 8.0, 5 mM EDTA, 1% β-mercaptoethanol). After centrifugation for 5 min in an Eppendorf centrifuge, the supernatant was transferred to a new tube and incubated with 5 units of N-glycosidase F (PNGase F; Roche Molecular Biochemicals) overnight at 37 °C. Samples were then analyzed on a 12% SDS gel under reducing conditions.

Mutagenesis—Mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using the pGEX-mADAM-2 disintegrin domain construct as template. Inserts were sequenced to verify that the desired mutation had, but that spurious mutations had not, been introduced.

Immunofluorescence Detection of Baculo D+C and Bacterial D Binding to Eggs—Zona-free eggs were preincubated with either purified baculo D+C for 30 min at 37 °C or with cleaved and purified bacterial D for 1 h at room temperature (subsequent procedures were performed at 4 °C to avoid internalization.) Eggs were then incubated with the 12CA5 mAb (100 μg/ml in PBS containing 1% BSA) for 45 min. After three washes with cold PBS/BSA the eggs were incubated with 25 μg/ml fluorescently labeled anti-mouse IgG for another 45 min. Eggs were then washed three times with cold PBS/BSA, mounted, and analyzed by confocal microscopy.

Sperm-Egg Binding Assays—Zona-free eggs were placed in 50-μl drops of M199 medium supplemented with 4 mg/ml BSA (fraction V, fatty acid free; Sigma). Eggs were pretreated with test proteins for 30 min prior to the addition of sperm. Capacitated sperm were added to a final concentration of 1 x 10^4 spermat and incubated for 30 min at 37 °C in a 5% CO₂ incubator. Eggs were washed three times in fresh medium, fixed in 4% glutaraldehyde, and mounted on glass slides for analysis by phase-contrast microscopy. 10–40 eggs were analyzed/condition, and the average number of sperm bound/egg was determined.

RESULTS

Expression of Disintegrin-like Domain-containing Constructs of Mouse ADAM 2 in Insect and Bacterial Cells—Our goal was to generate a recombinant protein to test the biological activity of the disintegrin-like domain of mADAM 2. Because both the disintegrin-like and cysteine-rich domains of mADAM 2 contain an odd number of cysteines (15 and 13, respectively; see Fig. 1A), because it has been hypothesized that these domains are structurally linked in the P-III SVMP, atrolysin A (3), and because a recombinant baculovirus encoding the D and C domains of atrolysin A was shown to have biological activity comparable to protein purified from snake venom (3), we initially generated a construct containing both the disintegrin-like (D) and cysteine-rich (C) domains (in tandem) of mADAM 2. In addition because of the large number of cysteines in these (extracellular) domains and the presence of sites for N-linked glycosylation (one in the disintegrin-like domain and three in the cysteine-rich domain; see Fig. 1A), we engineered our first construct to produce a secreted protein, and we expressed it in insect cells using a recombinant baculovirus system similar to that utilized for atrolysin A (3). Our construct encodes (from N to C terminus) a signal sequence (from melittin), the disintegrin-like and cysteine-rich domains of mADAM 2, a 9-amino acid tag from the influenza HA, and 8 His residues for purification over metal columns (Fig. 1A). This construct will be referred to as baculo D+C. Stocks of recombinant baculovirus encoding this construct were generated, and High Five cells were infected as described under “Experimental Procedures.” The medium from cells infected for 3 days with baculovirus encoding D+C was collected and analyzed on a Western blot using a mAb against the HA epitope tag. On reducing gels a prominent band of ~44 kDa was seen. (Fig. 1B, left lane). The predicted molecular mass of the baculo D+C protein is 28 kDa.

Sperm-Egg Fusion Assay—Eggs were preloaded with 0.5 μg/ml Hoechst 33342 stain (Sigma) in M199 medium supplemented with 4 mg/ml BSA (as above) for 30 min. Hoechst-stained eggs were transferred to fresh droplets of egg medium containing test proteins and incubated for another 30 min at 37 °C in a CO₂ incubator. Sperm were added to a final concentration of 1 x 10^4 spermat and incubated for an additional 30 min at 37 °C. The eggs were then washed five times by pipetting through a narrow bore glass pipette and fixed by adding glutaraldehyde to a final concentration of 0.25%. After 2–3 min, the eggs were mounted on a slide and immediately analyzed for fusion by fluorescence microscopy. The fusion-index (average number of sperm fused/egg) as well as the fusion rate (percentage of eggs fertilized) were determined (27).

Preparation of Protein-coated Fluorescent Beads—0.2 μm of yellow-green fluorescent sulfate-derivatized latex beads (Molecular Probes, Eugene, OR) were coated as follows: 10 μl of a 2% suspension of beads were incubated with 1 mg/ml purified test proteins for 2–3 h or overnight at 4 °C on an orbital platform shaker. The beads were then washed twice with PBS, quenched for 1 h with 0.2 mg/ml goat anti-rabbit IgG (Sigma), washed twice with PBS, and resuspended to 0.2% in egg medium. Alternatively, where indicated, the beads were first incubated with 0.4 mg/ml mAb 12CA5 in 20 μl of PBS for 2–3 h at 4 °C, quenched for 1 h with 0.2 mg/ml goat anti-rabbit IgG, and incubated with 10× concentrated medium containing the test protein for 3–4 h. The coated beads were then washed twice with PBS and resuspended to 0.2% in egg medium. Beads were used on the day of preparation and were sonicated 3 times for 5 s each at 4 °C immediately prior to use with a waterbath sonicator.

Bead- Egg Binding Assay—Zona-free eggs, prepared as indicated above, were placed in 20-μl drops of egg medium. Fluorescent beads coated with test proteins (as above) were added to give a final concentration of 0.02% and incubated for 1 h at 37 °C in a 5% CO₂ incubator. The eggs were gently agitated every 15 min. Eggs were then washed through three 100-μl drops of fresh medium, transferred to smaller drops, and visualized by confocal microscopy. Where indicated, eggs were pretreated with the indicated peptides or antibodies for 30 min at 37 °C. When anti-disintegrin loop antibodies or Fab fragments were used, protein-coated beads were first incubated with the antibodies or Fab fragments for 30 min in a 20-μl droplet, and then eggs were added.

D. Bigler, unpublished observation.
If all four sites for N-linked glycosylation are used, then the monomer molecular weight is expected to be $\sim 40-44$ kDa assuming a mass of $\sim 3-4$ kDa for each sugar side chain (28). To test the contribution of N-linked carbohydrate, we treated lysates containing baculo D+C with endoglycosidase F. As seen in Fig. 1C (lane 2) this treatment decreased the apparent mass of baculo D+C by $\sim 10$ kDa, resulting in a species that ran at $\sim 34$ kDa on reducing SDS gels. Production of baculo D+C in the presence of tunicamycin yielded a similar result (Fig. 1C, lane 4). The additional apparent mass of baculo D+C ($\sim 6$ kDa) may be because of O-linked glycosylation, other post-translational modifications, or to structural aspects of the protein (see below).

Baculo D+C was purified by affinity chromatography over a Talon column. A Western blot showing steps during the purification is seen in Fig. 1D. A silver-stained gel of the purified protein is seen in Fig. 1E, and a Western blot of the corresponding protein is seen in Fig. 1F. On reducing gels (Fig. 1, E and F, left lanes), a prominent band of $\sim 44$ kDa is seen. On nonreducing gels (Fig. 1, E and F, right lanes) bands of $\sim 36$, 72, and 88 kDa are seen. N-terminal sequencing of the purified protein revealed the sequence DPYVKMAVCVG, which represents the beginning of the disintegrin-like domain of mADAM 2 (in bold) plus four amino acids from the restriction sites used to subclone the polymerase chain reaction fragment into the expression vector. The yield of baculo D+C protein was $\sim 0.5$ mg/liter of culture medium.

Based on reports that disintegrin-like domain-containing constructs of mADAM 2 (22) and hADAM 15 (19) could be produced in bacteria, we also developed bacterial constructs encoding the disintegrin-like (and cysteine-rich) domains of mADAM 2. The construct encoding the entire ectodomain of mature mADAM 2 (i.e. disintegrin-like, cysteine-rich, EGF-like domain and pre-transmembrane domain spacer) made by Evans et al. (22) as a maltose-binding protein chimera ran as large multimeric aggregates near the top of the gel under nonreducing conditions and had to be reduced and “renatured” to manifest activity. In contrast, the construct of the disintegrin-like (D) domain of hADAM 15, made as a glutathione S-transferase chimera, ran as a single species on a nonreducing gel (19). We therefore employed the general strategy of Takada and co-workers (19). We constructed GST-D and GST-D+C (Fig. 2A) and expressed them in bacteria. The GST-D construct was readily produced in the DH5α strain of E. coli (Fig. 2B). Expression of GST-D+C in DH5α cells was very low; a better,
protein. The GST-D protein produced in bacteria also inhibited sperm-egg binding (Fig. 4C) and fusion (Fig. 4D) in a similar range of concentrations. The maximal extent of inhibition observed with baculo D+C (−75%) was higher than that observed for GST-D (−55%). GST-D+C and D protein released from GST-D showed a similar activity to GST-D (data not shown). Maximal inhibitory activity of both baculo D+C and GST-D was found with freshly prepared protein, with activity declining after about 1–2 weeks of storage at 4 °C.

Sequence Specificity of the Inhibitory Activity of the Disintegrin Domain of mADAM 2—To test the specificity of the observed inhibition of sperm-egg interactions caused by recombinant proteins encoding the disintegrin domain of mADAM 2, we engineered point mutations into the disintegrin loop of mADAM 2. The disintegrin loop sequence is CRLAQDECD- VTEYC. For our first set of mutants we changed each of the five charged residues within the loop to alanines; we also made a double alanine substitution for the two negatively charged residues located immediately upstream of the central cysteine. We chose these mutations because of the known involvement of charged residues in certain integrin-ligand interactions (30). As seen in Fig. 5A, the mutant in the ninth residue of the disintegrin loop, which we refer to as D9A, was significantly impaired (−80%) in its ability to inhibit sperm-egg binding. Individual alanine substitutions at the other negatively charged positions in the loop (D6A, E7A, and E12A) had little effect; the double mutation D6A/E7A showed ∼50% inhibitory activity of the wild type mADAM 2 disintegrin domain. An alanine substitution for the positively charged residue of the loop (R2A) did not significantly impair the inhibitory activity of the disintegrin domain. As a further test for the specificity of the inhibitory activity of the disintegrin domain of mADAM 2 we constructed an analogous GST-D domain from hADAM 12 (gift of Dr. U. Wewer, University of Copenhagen). The GST-D domain of hADAM 12 had a minimal effect on mouse sperm-egg binding (Fig. 5B).

Disintegrin-like Domain-containing Constructs of mADAM 2 Produced in Both Insect and Bacterial Cells Interact with the α₁ Integrin Subunit—We next explored the molecular basis for binding of the disintegrin-like domain-containing constructs to eggs. As seen in Fig. 6, fluorescent beads coated with baculo D+C bound to eggs in a manner that was specifically inhibited by the mADAM 2 disintegrin loop peptide (Fig. 6A) as well as by the function-blocking anti-α₁ mAb, GoH3 (Fig. 6B). Fluorescent beads coated with purified thrombin-released D protein produced in bacteria also bound to eggs. Binding of this bacterially produced protein was also inhibited by the function blocking mAb, GoH3 (Fig. 7A), as well as by an antibody against the disintegrin loop of mADAM 2 (Fig. 7B).

**Discussion**

ADAM 2 (fertilin β) is a glycoprotein that is essential for murine fertilization (31). Like all ADAMs, ADAM 2 contains a disintegrin-like domain with a clearly identifiable disintegrin loop (32–36). Several studies have shown that peptide analogues of the ADAM 2 disintegrin loop from guinea pig, mouse, and human (4, 5, 7, 8, 10, 11, 13) inhibit sperm-egg binding and fusion in vitro. In addition, three independent studies, using either peptide analogues of the ADAM 2 disintegrin loop (4, 6) or mADAM 2 purified from mouse sperm (37), have suggested that the disintegrin domain of mADAM 2 interacts with the egg integrin α₁β₁. However, another study, which employed a recombinant form of mADAM2 produced in bacteria as a maltose-binding protein chimera, concluded that mADAM 2 binds to a β₁ integrin on the egg but not to α₁β₁ (22). A goal of the present study was, therefore, to test whether recombinant mADAM 2 disintegrin domain-containing constructs can inter-
act with the \( \alpha_{6} \beta_{1} \) integrin. A second goal was to compare properties of recombinant disintegrin domain-containing constructs produced in insect cells and in bacteria. A third goal was to analyze the role of charged residues within the disintegrin loop.

The Disintegrin-like Domain of Mouse ADAM 2 Interacts with the \( \alpha_{6} \beta_{1} \) Integrin—To test the hypothesis that the disintegrin domain of mADAM 2 interacts with the \( \alpha_{6} \beta_{1} \) integrin, we produced recombinant disintegrin domain-containing constructs of mADAM 2 in insect cells using the baculovirus system as well as in E. coli. Our baculovirus construct encoded both the disintegrin-like (D) and cysteine-rich (C) domains of mADAM 2 and was purified from the medium of baculovirus-infected cells; it was similar to a construct of the P-III SVMP, atrolysin A, described by Fox and co-workers (3). Our bacterial constructs encoded the D domain of mADAM 2 fused to GST; it was similar to a construct of hADAM 15 prepared by Takada and co-workers (19).

Baculo D+C as well as GST-D inhibited sperm-egg binding and fusion in vitro (see below). Fluorescent beads coated with
either protein bound to the egg, and in both cases, bead-binding was inhibited by the function-blocking anti-\(\alpha_6\) integrin mAb, GoH3, but not by the nonfunction-blocking anti-\(\alpha_6\) integrin mAb, J1B5.\(^6\) Therefore, as shown for mature mADAM 2 purified from sperm (21), binding (to eggs) of recombinant disintegrin-like domain-containing constructs of mADAM 2 produced in both insect cells and in bacteria, can be specifically inhibited by a function-blocking anti-\(\alpha_6\) integrin mAbs.

The concentration of GoH3 needed to inhibit binding of mature mADAM 2 isolated from sperm (21) or recombinant mADAM 2 disintegrin domain-containing constructs is 100 \(\mu\)g to 200 \(\mu\)g/mL. This is 10-fold higher than the concentration of GoH3 needed to inhibit \(\alpha_{5}\)-mediated cell adhesion to laminin (38). We consider three possibilities to explain these observations. The first is that the ADAM 2 D domain interacts with a different isoform of \(\alpha_6\) than the one that binds laminin, perhaps an alternate splice form in its ectodomain (39). The second, as suggested by our recent findings (21, 37), is that the ADAM 2 D domain interacts with an alternate state of \(\alpha_6\). A third is that ADAM 2 interacts with an \(\alpha_6\)-like integrin on the egg. We propose that the presumptive alternate \(\alpha_6\) has a lower affinity for GoH3 than the classic form and hence that higher concentrations of GoH3 are needed to inhibit its function. In recent work we have provided evidence that the tetraspan integrin-associated protein CD9 is involved in binding mADAM 2 (37). Hence we are presently considering three models for how the mADAM 2 D domain may interact with a complex of \(\alpha_6\) and CD9 on the egg (Fig. 8). The D domain may interact with \(\alpha_6\) (Fig. 8A), with sites on both \(\alpha_6\) and CD9 (Fig. 8B), or with CD9 (Fig. 8C) within the complex. Current experiments are aimed at testing these possibilities.

**Comparison of Baculo D+C with the Disintegrin-like Domain-containing Construct of Atrolysin A Produced in Insect Cells**—We modeled our first construct, baculo D+C, after a construct of the P-III SVMP atrolysin A, a construct that has been shown to inhibit platelet aggregation (3). We chose to use a eukaryotic expression system for our first construct because the D and C domains of ADAMs are predicted to be glycosylated and because they each contain unusually high and odd numbers of cysteines. In addition, it has been shown that multidomain proteins fold better in eukaryotic, than in prokaryotic, systems (40). Based on these considerations, we chose to purify the baculo D+C that was secreted into the culture medium, even though a significant fraction was retained within the cells.\(^7\) mADAM 2 baculo D+C maximally inhibited sperm-egg binding and fusion in the concentration range of 0.5 to 5 \(\mu\)M. The atrolysin A D+C construct inhibited platelet aggregation with an \(\text{IC}_{50}\) of 0.3–0.4 \(\mu\)M. From our data (Fig. 4, A and B), baculo D+C (mADAM 2) appears to have a potency similar to that of recombinant atrolysin A D+C. The potencies of both mADAM 2 D+C and atrolysin A D+C produced in insect cells are considerably higher than those of peptide analogues of their respective disintegrin loops, which are inhibitory at \(\sim 200–500 \mu\)M (3, 4, 7, 10, 11, 41).

**Comparison of Baculo D+C with GST-D and other ADAM Disintegrin-like Domain-containing Constructs**—We also produced mADAM 2 disintegrin-like domain-containing constructs in bacteria as GST chimeric proteins. Of note, and possibly reflecting the difficulty of folding multidomain proteins in bacterial systems (40), GST-D+C was protease sensitive, was not produced in high yield, and proved difficult to purify. The GST-D construct was, however, readily produced and purified. GST-D inhibited sperm-egg binding and fusion with good potency (maximal inhibition with 1–5 \(\mu\)M protein).

Although maximally inhibitory in a similar range of concentrations, the maximum extent of inhibition seen with GST-D was somewhat lower than that seen with baculo D+C. The facts, however, that GST-D demonstrated a high level of potency and ran as a single species on both reducing and nonreducing SDS gels may indicate that the GST protein facilitated folding of the downstream D domain, perhaps during purification over glutathione-agarose columns (i.e. under reducing conditions).

It is difficult to compare the potency of the mADAM 2 GST-D domain produced here with the previously described maltose-binding protein chimera of the mature fibrin \(\beta\) extracellular domain (\(\beta\)-DCE), because data for only two concentrations of \(\beta\)-DCE were reported: \(\sim 6 \mu\)M \(\beta\)-DCE inhibited sperm-egg binding by \(-50\%\) and 3 \(\mu\)M \(\beta\)-DCE showed no significant inhibitory effect (18). Hence, the GST-D construct (Fig. 4C) appears to be a more potent inhibitor than the maltose-binding protein chimera.

The only other ADAM that has, to date, been shown to interact with an integrin is hADAM 15, which is unique in containing an RGD sequence in its disintegrin loop. The hADAM 15 D domain made as a GST chimera, as well as a construct containing the entire extracellular domain of hADAM 15 fused to the Fc portion of IgG, were shown to support binding in solid phase cell binding assays to the \(\alpha_{5}\beta_3\) integrin. The Fc chimera protein also interacted with \(\alpha_6\beta_1\). However, the potencies of these ADAM 15 constructs, for example in inhibiting integrin-mediated binding to fibronectin, were not reported (19, 20).

**Sequence Specificity of the Inhibitory Activity of the mADAM 2 Disintegrin Domain**—As expected, the interaction of our mADAM 2 disintegrin domain-containing constructs with eggs was inhibited by a peptide analogue of and by an antibody against the disintegrin loop. We therefore employed a mutagenesis approach to begin to assess the sequence require-

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\(^6\) We recently presented a preliminary demonstration of the inhibitory effect of GoH3 on binding of the bacterially produced D protein (37).

\(^7\) D. Bigler, M. S. Chen, and J. M. White, unpublished results.
Fig. 7. Binding of beads coated with cleaved bacterial D to eggs: effects of anti-integrin α6 antibodies and an anti-disintegrin-loop antibody. Fluorescent beads were coated with D protein cleaved and purified from GST-D. A, eggs were pretreated with 0.2 mg/ml mAb GoH3 or mAb J1B5. B, beads were pretreated with 0.1 mg/ml of either a control antibody (Ab) (anti-fertilin α cytoplasmic tail) or an anti-disintegrin loop Ab, or with 0.1 mg/ml of Fab fragments from rabbit IgG (control Fab) or Fab fragments from the anti-disintegrin-loop Ab (anti-disintegrin loop Fab).

Fig. 8. Model for possible modes of interaction between the mADAM 2 D domain and an α6CD9 complex on the egg. We denote α6 in quotations to indicate that ADAM 2 may interact with an alternate form of α6 (or an α6-like integrin) on the egg (see “Discussion”). A, ADAM 2 interacts with only α6. B, ADAM 2 interacts with sites on both α6 and CD9. C, ADAM 2 interacts with only CD9.

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Note Added in Proof—Female mice that lack CD9 (see Fig. 8) show severely reduced fertility because of a block to sperm-egg fusion (Miyou, K., Yamada, G., Yamada, S., Hasuwa, H., Nakamura, Y., Ryu, F., Suzuki, K., Kosei, K., Inoue, K., Ogura, A., Okabe, M., and Makeda, E. (2000) Science 287, 321–324 and Le Naour, F., Rubinstein, E., Jasmin, C., Prenant, M., and Boucheix, C. (2000) Science 287, 319–321).

REFERENCES

1. Black, R. A., and White, J. M. (1998) Curr. Opin. Cell Biol. 10, 654–659
2. Schindlaurf, J., and Blobel, C. P. (1999) J. Cell Sci. 112, 3603–3617
3. Jia, L.-G., Wang, X.-M., Shannon, J. D., Bjarnason, J. H., and Fox, J. W. (1997) J. Biol. Chem. 272, 13094–13102
4. Almeida, E. A. C., Huovila, A.-P. J., Sunderland, A. E., Stephens, L. E., Calarco, P. G., Shaw, L. M., Mercurio, A. M., Sonnenberg, A., Primakoff, P., Myles, D. G., and White, J. M. (1995) Cell 81, 1095–1104
5. Bronson, R. A., Fusi, F. M., Calzi, F., Doldi, N., and Ferrari, A. (1999) Mol. Hum. Reprod. 5, 433–440
6. Chen, H., and Sampson, N. S. (1999) Chem. Biol. 6, 1–10
7. Evans, J. P., Schultz, R. M., and Kopf, G. S. (1995) J. Cell Sci. 108, 3267–3278
8. Giebisch, P. M., Ford, W. C. L., and Hall, L. (1997) Int. J. Androl. 20, 165–176
9. Linder, B., and Heinlein, U. A. O. (1997) Dev. Growth Differ. 39, 243–247
10. Myles, D. G., Kimmel, L. H., Blobel, C. P., White, J. M., and Primakoff, P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4195–4198
11. Pylock, A., Yuan, H., Galilgan, E., Primakoff, P., Myles, D. G., and Sampson, N. S. (1997) Bioorg. Med. Chem. Lett. 7, 1053–1058
12. Shilling, F. M., Kratczschmar, J., Cai, H., Weskamp, G., Gayko, U., Leibow, J., Myles, D. G., Nuccitelli, R., and Blobel, C. P. (1997) Dev. Biol. 186, 155–164
13. Yuan, H., Primakoff, P., and Myles, D. G. (1997) J. Cell Biol. 137, 115–122
14. Blobel, C. P., Myles, D. G., Primakoff, P., and White, J. M. (1990) J. Cell Biol. 111, 69–78
15. Humarina, G. R., Koppel, D. E., and Myles, D. G. (1997) Dev. Biol. 191, 146–159
16. Lum, L., and Blobel, C. P. (1997) Dev. Biol. 191, 131–145
17. Phelps, B. M., Koppel, D. E., Primakoff, P., and Myles, D. G. (1990) J. Cell Biol. 111, 1839–1847
18. Evans, J. P., Schultz, R. M., and Kopf, G. S. (1998) Biol. Reprod. 59, 145–152
19. Zhang, X.-P., Kamata, T., Yokoyama, K., Puzon-McLaughlin, W., and Takada, Y. (1998) J. Biol. Chem. 273, 7345–7350
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20. Nath, D., Slocombe, P. M., Stephens, P. E., Warn, A., Hutchinson, G. R., Yamada, K. M., Docherty, A. J. P., and Murphy, G. (1999) J. Cell Sci. 112, 579–587
21. Chen, M. S., Almeida, E. A. C., Huovila, A.-P. J., Takahashi, Y., Shaw, L. M., Mercurio, A. M., and White, J. M. (1999) J. Cell Biol. 144, 549–561
22. Evans, J. P., Kopf, G. S., and Schultz, R. M. (1997) Dev. Biol. 187, 79–93
23. Wolfsberg, T. G., Straight, P. D., Gerena, R. L., Huovila, A.-P. J., Primakoff, P., Myles, D. G., and White, J. M. (1995) Dev. Biol. 169, 378–383
24. Tessier, D. C., Thomas, D. Y., Khouri, H. E., Lahiberte, F., and Vernet, T. (1991) Gene (Amst.) 98, 177–183
25. Balliet, J. W., Berson, J., D'Cruz, C. M., Huang, J., Crane, J., Gilbert, J. M., and Bates, P. (1999) J. Virol. 73, 3054–3061
26. Gilbert, J. M., Bates, P., Varmus, H. E., and White, J. M. (1994) J. Virol. 67, 6889–6892
27. Cosnard, S. A., Naaby-Hansen, S., Shetty, J., Shibahara, H., Chen, M., White, J. M., and Herr, J. C. (1999) Dev. Biol. 207, 334–349
28. Lewis, V., Green, S. A., Marsh, M., Vihko, P., Helenius, A., and Mellman, I. (1985) J. Cell Biol. 100, 1839–1847
29. Wilson, N. F., and Snell, W. J. (1998) Trends Cell Biol. 8, 93–96
30. Bergelson, J. M., and Hemler, M. E. (1995) Curr. Biol. 5, 615–617
31. Cho, C., O’Dell Bunch, D., Faure, J. E., Goulding, E. H., Eddy, E. M., Primakoff, P., and Myles, D. G. (1998) Science 281, 1857–1859
32. Bigler, D., Chen, M., Waters, S., and White, J. M. (1997) Trends Cell Biol. 7, 220–225
33. Evans, J. P. (1999) Front. Biosci. 4, 114–131
34. Myles, D. G., and Primakoff, P. (1997) Biol. Reprod. 56, 320–327
35. Wasserman, P. M. (1999) Cell 96, 175–183
36. Wolfsberg, T. G., and White, J. M. (1996) Dev. Biol. 180, 389–401
37. Chen, M. S., Tung, K. S. K., Cosnard, S. A., Takahashi, Y., Bigler, D., Chang, A., Yamashita, Y., Kincade, P. W., Herr, J. C., and White, J. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11830–11835
38. Chen, L., Shick, V., Matter, M. L., Laurie, S. M., Ogle, R. C., and Laurie, G. W. (1997) Am. J. Physiol. 272, L494–L503
39. Delwel, G. O., Kuikman, I., and Sonnenberg, A. (1995) Cell Adhes. Comm. 3, 143–161
40. Netzer, W. J., and Hartl, F. U. (1997) Nature 388, 343–349
41. McClane, M. A., Marcinkiewicz, C., Vijay-Kumar, S., Wierzchiza-Patynowski, I., and Niewiarowski, S. (1998) Proc. Soc. Exp. Biol. Med. 219, 109–119
42. Blobel, C. P., and White, J. M. (1992) Curr. Opin. Cell Biol. 4, 760–765
Sequence-specific Interaction between the Disintegrin Domain of Mouse ADAM 2 (Fertilin \( \beta \)) and Murine Eggs: ROLE OF THE \( \alpha 6 \) INTEGRIN SUBUNIT
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