Species-Specific Inhibition of Fertilization by a Peptide Derived from the Sperm Protein Bindin

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The sperm protein bindin is responsible for the species-specific adhesion of the sperm to the egg. The regions of the bindin molecule responsible for forming the contact between the sperm and the egg were investigated by measuring the ability of peptides representing various regions of the bindin sequence to inhibit fertilization. Twenty-four peptides were studied: 7 based on the *Strongylocentrotus purpuratus* bindin sequence, 11 based on the *S. franciscanus* bindin sequence, and 6 control peptides. Values for the concentration of peptide required to inhibit 50% of the productive sperm contacts (IC_{50}) were extracted from experimental measurements of the extent of fertilization in the presence of various concentrations of these peptides. The IC_{50} value averaged 220 μM for the control peptides. Active peptides representing certain specific subregions of the bindin sequence displayed IC_{50} values <10% of the average value for control peptides, and the IC_{50} for the most potent of the peptides tested was only ~1% of the control peptide value (IC_{50} = 2.2 μM). Furthermore, we found that a peptide representing a particular region of the *S. franciscanus* bindin sequence that differs from the *S. purpuratus* bindin sequence inhibits fertilization species specifically. For the reaction of *S. purpuratus* sperm and eggs, the IC_{50} of this peptide was ~120 μM, whereas for the reaction of *S. franciscanus* sperm and eggs it was only 8.6 μM. These results demonstrate that a few specific regions of the bindin molecule are involved in the sperm-egg contact and that certain of these regions mediate the species specificity of the interaction in a sequence-specific manner.

INTRODUCTION

Fertilization in sea urchins involves a series of interactions between the sperm and the egg (for reviews see Vacquier and Moy, 1978; Glabe et al., 1982; Rossignol et al., 1984a; Minor et al., 1989). The specificity of fertilization can be due to interspecies incompatibility at any of three different steps in the fertilization pathway. These steps are chemotaxis of the sperm to the egg (Ward et al., 1985), induction of the acrosome reaction (Summers and Hylander, 1975; Segall and Lennarz, 1979), and binding of the sperm to the egg

(Summers and Hylander, 1975; Glabe and Vacquier, 1977). Summers and Hylander (1975) determined that in most (9 of 11) cases the species specificity of fertilization is due to the failure of sperm to bind productively to the eggs of other species. This is the case for the species *Strongylocentrotus purpuratus* and *S. franciscanus*, where the specificity of fertilization has been shown to be due to the specific interaction of the sperm adhesive protein bindin, with receptors on the surface of the egg (Glabe and Vacquier, 1977, 1978; see also Minor et al., 1991). A glycoprotein egg surface receptor has been identified recently by Foltz and Lennarz (1990). They showed that a soluble proteolytic fragment of this receptor will block fertilization species specifically, and this fragment interacts

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differentially in vitro with the bindins of *S. purpuratus* as opposed to those of other species. Before activation, the bindin is packaged as a insoluble granule in the sperm acrosome. When the sperm is activated, the acrosome everts, and the bindin is exposed. It remains associated with the sperm membrane and thus binds the sperm head to the receptors on the surface of the egg. Bindin may be involved in subsequent steps of membrane fusion as well (Glabe, 1985a,b; Kennedy *et al.*, 1989).

The bindin protein from *S. purpuratus* was purified and partially sequenced (Vacquier and Moy, 1977, 1978; Sasagawa and Walsh, personal communication). Knowledge of the partial sequence permitted the isolation of an *S. purpuratus* bindin cDNA, and the clone was used to show that the bindin gene is indeed transcribed only in testes (Gao *et al.*, 1986), relatively late in spermatogenesis (Cameron *et al.*, 1990; Nishioka *et al.*, 1990). *S. purpuratus* bindin is synthesized initially as a 51-kDa precursor polypeptide that is cleaved to yield the 24-kDa acrosomal bindin from its C-terminus, plus a 27-kDa N-terminal protein of unknown function (Gao *et al.*, 1986). In the spermatocyte the mRNA is immediately translated, and the bindin is packaged into the acrosomal vesicle (Nishioka *et al.*, 1990). Bindin cDNAs representing the complete open reading frame also have been cloned and sequenced from three other species of sea urchin: *Arbacia punctulata* (Glabe and Clark, 1991), *S. franciscanus*, and *Lytechinus variegatus* (Minor *et al.*, 1991). Like the *S. purpuratus* bindin, all of these bindins are cleaved from the C-termini of much larger precursors. The mature bindin proteins differ in several regions of the sequence, and some or all of these differences must be responsible for the species-specific activity of bindin.

In this study we attempted to determine whether there are particular elements of the bindins of *S. purpuratus* and *S. franciscanus* that are responsible for species-specific sperm adhesion. Peptides were synthesized to match various regions of the bindins of both species and added at various concentrations to fertilization reactions carried out with limiting amounts of sperm. If such peptides occupy sites in the receptor that would normally be occupied by the corresponding regions of the acrosomal bindin protein, fertilization would be inhibited, i.e., if the bindin-peptide/receptor complex is sufficiently stable and if presence of the peptide would sterically prevent the bindin from occupying the receptor. Some of the peptides we tested were indeed found to block fertilization at relatively low concentrations. These results imply that bindin interaction with the egg receptor involves a direct sequence dependent recognition process. It follows that the species specificity of bindin function must be due to the formation of molecular contacts between the receptor and regions of the bindin molecule that are distinct in each species.

MATERIALS AND METHODS

Peptides

Peptides were synthesized and characterized in the Microchemical Facility at Caltech. All peptides were >80% pure (as judged by high-performance liquid chromatography profiles). All peptides were checked for amino acid content, and those that differed from the expected molar ratios were sequenced (the errors in synthesis of peptides SpA and SfN were detected in this way). Peptide SfQ was further purified by preparative fast protein liquid chromatography.

Peptides derived from the *S. purpuratus* sequence are shown in Figure 1; those derived from the *S. franciscanus* sequence are shown in Figure 2. Six peptides were selected for control experiments on the basis that they showed no significant sequence similarity to bindin. The sequences of these peptides are as follows (from N-terminus to C-terminus): CP1 (ASRDCQNGAV), CP2 (QNGGICIDGINGYT), CP3 (LNISEVKSQFYRYPFICVVKN), CP4 (IWDNWGVDKFDVYLSRRKC), CP5 (KPPSPISEAPRTLASC), and CP6 (MAKTAMAYKEKMKEL-SMLSLIC).

Inhibition of Fertilization Assay

All work was done in a 16°C room. Gametes were collected by either electric shock or by intracoelomic injection of 0.5 M KCl. The eggs were dejellied by washing in Millipore-filtered sea water (MPFSW; Millipore, Bedford, MA) titrated to pH 5 by the addition of citric acid, washed in MPFSW, and then returned to MPFSW in a 800 egg/ml suspension. Six hundred microliters of this suspension was placed in each well of a cell culture plate (model 3047; Falcon, Lincoln Park, NJ). Peptide solutions were prepared in MPFSW at a concentration 2.5-fold higher than the test concentration, and either HCl or NaOH was added to bring the solutions to pH 8 (when necessary). The amount of peptide to be used was determined gravimetrically, assuming that the peptide samples were 100% pure. Four hundred microliters of these solutions were added to the eggs. After a 1-h incubation with peptide, 40 μl of a freshly prepared sperm dilution was added to the eggs. The sperm dilution was prepared by diluting dry sperm in MPFSW containing 0.005% bovine serum albumin (BSA, Fraction V; Sigma Chemical, St. Louis, MO). The dry sperm was titrated to determine the dilution necessary to achieve 75% fertilization in the controls. The eggs were fixed 5 min after the addition of sperm by the addition of an equal volume of 2% glutaraldehyde (Sigma) in MPFSW. The positions of the treatments and controls in the wells were randomized, and the plates were coded before scoring. Over 100 eggs were counted for each experimental data point. Eggs were scored as fertilized on the basis of the elevation of the fertilization envelope when viewed at 100X in an inverted microscope. Eggs were treated with peptide, fertilized, fixed, and scored all in the same well to avoid the preferential loss of unfertilized eggs that occurs when eggs are transferred.

Definition of Terms

Let C be the fraction of eggs fertilized in the control (no peptide added)

$$C = \frac{\text{number eggs fertilized (no peptide)}}{\text{total number of eggs counted}} \qquad (1)$$

Let E be the fraction of eggs fertilized when peptide is added

$$E = \frac{\text{number eggs fertilized (peptide present)}}{\text{total number of eggs counted}} \qquad (2)$$

Then the fraction of eggs whose fertilization was blocked by the peptide, B, is given by

$$B = C - E \qquad (3)$$

Thus, the fraction of fertilizations inhibited by the peptide, FFI, is (from Eq. 3)

$$FFI = \frac{B}{C} = 1 - \frac{E}{C} \tag{4}$$

An expression was derived to quantify the fraction of productive sperm contacts inhibited, F. This was necessary for comparison of experiments that had differing degrees of fertilization in the control. The expression for F is derived here based on the assumption that the distribution of the sperm among the eggs is approximated by the Poisson distribution. In this case, the percentage of eggs in the sample that receive R active sperm is

$$\text{fraction of eggs with R active sperm} = \frac{\mu^R}{R!e^\mu} \tag{5}$$

where μ is the mean number of active sperm per egg calculated from the Poisson zero class (i.e., $1 - C$, from Eq. 1), or

$$\mu = \ln\frac{1}{1 - C} \tag{6}$$

The fraction of the eggs with R active sperm whose fertilization is inhibited by peptide is given by the inhibitory factor, F, to the power R. For example, if F = 0.5, then of the eggs with only one sperm, one-half (F[1]) of the eggs will not fertilize. Of the eggs with two sperm, only one-quarter (F[2]) will not fertilize because both sperm must be inhibited to keep the egg from being fertilized. One-eighth (F[3]) of the eggs with three sperm will not fertilize, one-sixteenth (F[4]) of the eggs with four sperm will not fertilize, etc. The fraction B of eggs in the entire sample whose fertilization is inhibited is given by

$$B = \sum_{R=1}^{\infty}\left(\frac{\mu^R}{R!e^\mu}(F^R)\right) \tag{7}$$

This can be rearranged to

$$B = (e^{(\mu F - \mu)})\left(\sum_{R=1}^{\infty}\frac{(\mu F)^R}{R!e^{\mu F}}\right) \tag{8}$$

The right-hand term is the Poisson series summation minus the zero class, so Eq. 8 can be expressed as

$$B = (e^{(\mu F - \mu)})(1 - e^{-\mu F}) \tag{9}$$

Substituting Eq. 3 for B and Eq. 6 for μ, Eq. 9 can now be expressed as

$$F = 1 - \frac{\ln(1 - E)}{\ln(1 - C)} \tag{10}$$

This expression allows the fraction of sperm contacts inhibited to be determined from the experimental data. Note that Eq. 10 states that F is independent of sperm concentration.

Hill Analysis of Micromolar Versus F Data

A single determination of peptide inhibition consists of a set of data points of the inhibitory effect F of a peptide at a given micromolar concentration (e.g., see Figure 3). To fit these data and extract the value for the amount of peptide necessary to achieve 50% inhibition of sperm contacts, a Hill analysis of the data was used (see, e.g., Marshall, 1978). This analysis assumes peptides (I) are filling sperm binding sites in an equilibrium manner. If n peptides are required to inactivate a sperm binding site, then the experimental data can be fit to the Hill equation

$$\log\frac{F}{1 - F} = n\log(I) - \log(K_I) \tag{11}$$

where K_I is an equilibrium constant. In a plot of $\log(I)$ versus $\log(F/(1 - F))$ (Hill plot), n is the slope and $-\log(K_I)$ is the Y-intercept. The concentration of peptide, I (in μM), necessary for 50% inhibition of sperm contacts (IC_{50}), i.e., when F = 0.5, is given by

$$IC_{50} = (K_I)^{(1/n)} \tag{12}$$

In this work a single determination of the value of n for a given peptide consists of at least four data points, of which the least inhibited has $0 < F < 0.75$ and the most inhibited has $0.75 < F < 1$. The value F = 0.75 is an important value for these titrations to span. When F = 0.75, FFI $\approx$ 0.5 (Eq. 4), which is where the highest transition from fertilized to unfertilized eggs is taking place. Of these determinations, only those that had n $\geq$ 1 were used for the determination of IC_{50}. The majority of determinations for all of the peptides had n > 1, and the occasional titration curves yielding values of n < 1 show little dose-response relationship due to high data scatter. These titrations are therefore likely to be misleading for determining IC_{50} and have been omitted for the determinations of IC_{50} only.

The data for each determination were individually fit by least-squares linear regression to the Hill equation, and values for n and IC_{50} were extracted. For all determinations, the fraction of eggs fertilized in the control was $0.50 < C \leq 0.90$ and averaged 0.75. Because the control fertilizations were performed within a narrow interval, the relative difference between fitting the peptide inhibition data to F or FFI was minimal. When the inhibition of fertilization data is fit to FFI rather than F, the values for n average 2% higher and the values for IC_{50} average 40% higher. The relative values for IC_{50} are largely unaffected when fit to FFI rather than F, because all of the values increase to the same degree when fit to FFI.

Statistical Test of Species Differences in IC50

Differences in IC_{50} for a given peptide between species were tested for statistical significance using the approximate t-test for the equality of two means (Sokal and Rohlf, 1981). Peptide SfR is the only peptide that has a significant difference in IC_{50} between the two species ($\alpha < 0.05$) (the species difference in IC_{50} for this peptide is also significant [$\alpha < 0.05$] if the determinations where n < 1 are also included).

Control Experiment Protocols

Observation of Eggs Through Cleavage. The experiment is performed as in the inhibition assay, and all of the treatments are performed in duplicate. One of the treatments is fixed 5 min postsperm addition as in the inhibition assay, but the other treatment is fixed 2 h after the addition of sperm. All of the treatments are scored for unfertilized eggs, fertilized eggs, and fertilized embryos that have cleaved.
Treatment of Peptide Inhibited Eggs with A23187. The experiment is performed as in the inhibition assay, and all of the treatments are performed in duplicate. One of the treatments is 5 min postsperm addition as in the inhibition assay, but the other treatment has no sperm added and is fixed 5 min after the addition of 2.5 μM A23187 (Sigma). All of the treatments are scored for eggs with and without an elevated fertilization membrane.
Toxicity of Peptide on Sperm. In this assay, the peptide is added to the sperm dilution before its addition to the eggs. The sperm and peptide are coincubated for 5 min and then added to the eggs (which have no peptide added). Forty microliters of the sperm-peptide mix were added to the eggs as in the inhibition of fertilization assay, and the assay is completed as described above for the inhibition assay.
Toxicity of Peptide on Eggs. After the 1-h coincubation as in the inhibition assay, the MPFSW containing peptide is removed by aspiration and replaced with fresh MPFSW. After times varying from 1 min to 1 h, sperm were added, and the assay proceeds as described above in the inhibition assay.
Evaluation of the Acrosome Reaction. The extent of the acrosome reaction in sperm treated with peptide was monitored by a modification of the phenyl bead method of Yamada and Aketa (1988). This method
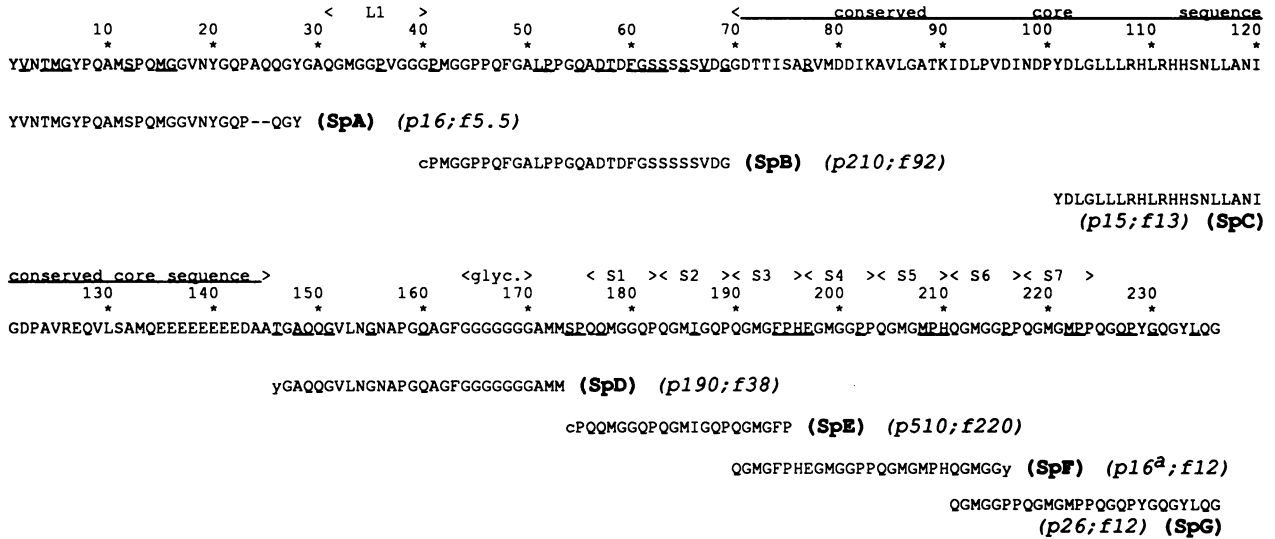
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YVNTMGYPQAMSPQMGGVNYGQP--QGY (SpA)  (p16;f5.5)
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                                                                     (p15;f13)  (SpC)
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conserved core sequence >                    <glyc.>      < S1  >< S2  >< S3  >< S4  >< S5  >< S6  >< S7  >
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           yGAQQGVLNGNAPGQAGFGGGGGGGAMM (SpD)  (p190;f38)
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                        cPQQMGGQPQGMIGQPQGMGFP (SpE)  (p510;f220)
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                               QGMGGPPQGMGMPPQGQPYGQGYLQG
                                   (p26;f12)  (SpG)
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Figure 1. *Strongylocentrotus purpuratus* bindin sequence and synthesized peptides. The *S. purpuratus* bindin protein sequence (Gao *et al.*, 1986) is shown, numbered every 10 amino acids. Features of bindin are shown above the sequence and are defined in Minor *et al.* (1991). "L1" marks a copy of the long consensus sequence, "glyc" indicates a polyglycine stretch, and "S1-S7" mark copies of the short consensus sequence. Underlined amino acids are those that differ between the bindins of *S. purpuratus* and *S. franciscanus*. Peptides (SpA to SpG) synthesized to match portions of the bindin protein are shown beneath the regions that they match and are labeled in boldface. Lower-case letters in the peptide sequences indicate amino acids that were added during synthesis that do not match the bindin sequence. The two dashes in the SpA sequence indicate that this peptide was synthesized without the amino acids AQ. The values in italics indicate the concentration (in μM) of peptide necessary to inhibit sperm contacts 50%. The value "p" indicates the effect of the peptides on *S. purpuratus* fertilizations; the value "f" indicates the effect on *S. franciscanus* fertilizations. [a]This value is from one experiment only.

takes advantage of the fact that only acrosome reacted sperm stick to phenyl Sepharose beads. Phenyl Sepharose CL-4B beads were washed and equilibrated in MPFSW. Egg jelly was prepared as described by Vacquier (1986). Experiments were performed in 13 × 100-mm glass test tubes. Five hundred microliters of MPFSW with or without peptide and with or without egg jelly (1:8 dilution) were added to the tubes. Fifty microliters of a 7% suspension (vol/vol) of phenyl beads were added to the tubes. Dry sperm was diluted 10 μl to 3 ml in MPFSW with 10 mM NaHCO$_3$ (Vacquier, 1986) and 0.005% BSA (Sigma). One hundred microliters of this dilution was added to the tubes. One minute later, the sperm/beads were fixed with 8 ml of 2% glutaraldehyde in MPFSW. The beads were spun down (clinical centrifuge on low), and the supernatant was removed by aspiration. The beads were resuspended in 9 ml of fixative, spun down, and the supernatant was again removed by aspiration. This wash was repeated once more. The pelleted beads were resuspended, and the number of sperm bead perimeter (when the focal plane of the microscope was on the equator of the bead) was counted at 320× under phase contrast.

Time Course of Peptide Inhibition. Peptide is added to the eggs as in the inhibition of fertilization assay, but the peptide and eggs are coincubated for times ranging from 1 min to 1 h. The eggs are then fertilized and fixed as described above in the inhibition assay.

Peptide Effect on Sperm-Egg Binding. The effects of peptides on sperm-egg binding were monitored by experiments modeled after those performed by Vacquier *et al.* (1973), Vacquier and Payne (1973), Vacquier *et al.* (1979), and Kinsey *et al.* (1980). Experiments were performed in 13 × 100-mm glass test tubes. Four hundred microliters of MPFSW containing 1,000 dejellied eggs was added to each test tube. One hundred microliters of the test solution was added to the eggs (the test solution was either MPFSW as a control or MPFSW containing peptide). Dry sperm was diluted 10 μl to 5 ml in MPFSW with 10 mM NaHCO$_3$ (Vacquier, 1986) and 0.005% BSA (Sigma). Five hundred microliters of the sperm dilution was added to the 500 μl egg-peptide suspension in the test tube. Twenty-five seconds later,

the sperm/eggs were fixed with 8 ml of 3% formaldehyde in MPFSW. The eggs were spun down (clinical centrifuge on low), and the supernatant was removed by aspiration. The eggs were resuspended in 9 ml of fixative, spun down, and the supernatant was again removed by aspiration. This wash was repeated once more. The eggs were resuspended and the number of sperm per egg perimeter at the equator was counted at 320× under phase contrast.

RESULTS

Peptide Inhibition of Fertilization

The peptides used in this study are shown in Figure 1 (*S. purpuratus*-derived peptides), Figure 2 (*S. franciscanus*-derived peptides), and in MATERIALS AND METHODS (control peptides). Data illustrating the inhibitory effects of peptides SfO and SpE on both *S. purpuratus* and *S. franciscanus* fertilizations are shown in Figure 3. Peptide SpE does not inhibit the fertilization of either species very well (mean 50% inhibition concentration is 220 μM for *S. franciscanus* eggs and ~510 μM for *S. purpuratus* eggs), whereas peptide SfO inhibits fertilization of both species at ~100-fold lower concentration (mean 50% inhibition concentration is 3.8 μM for *S. franciscanus* eggs and 3.6 μM for *S. purpuratus* eggs). Each experiment was separately fit to the Hill equation (Eq. *11*, MATERIALS AND METHODS), and an example is shown in Figure 4. The regression is used to obtain the slope of the line, n, and the concentration at which 50% inhibition of sperm contacts occurs, IC$_{50}$.
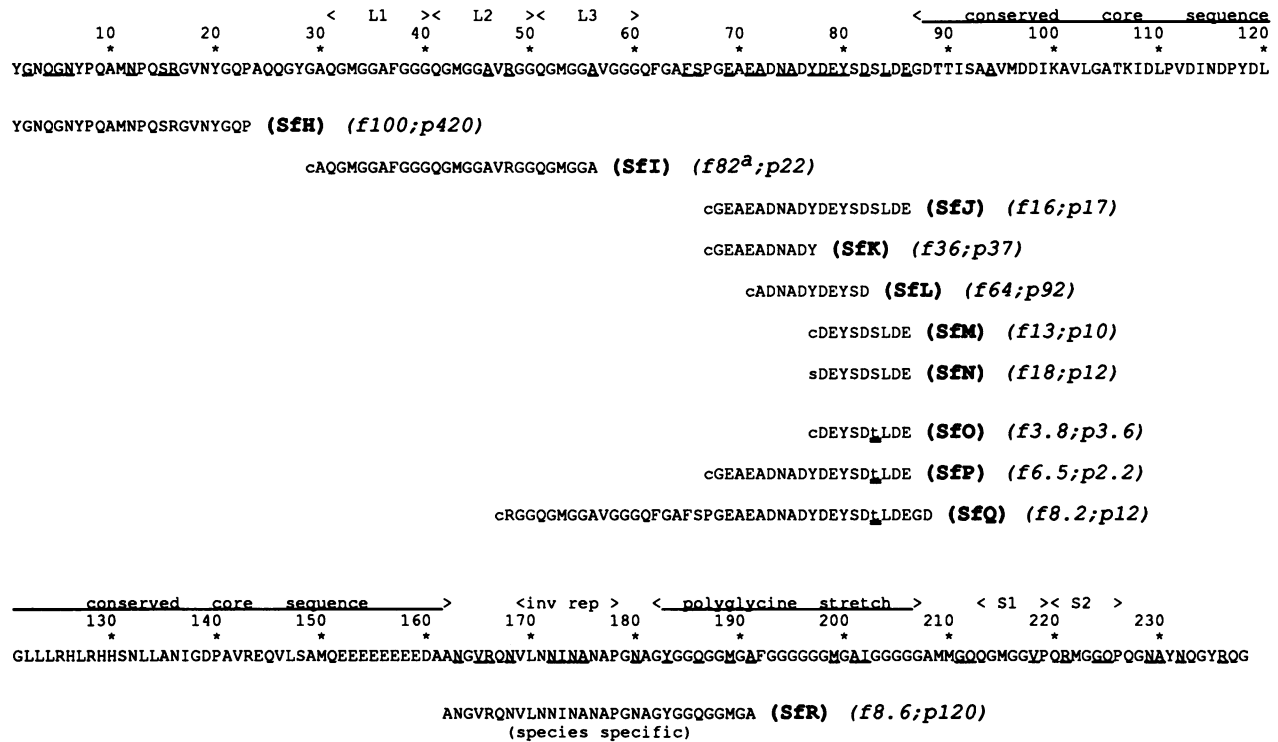
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YGNQGNYPQAMNPQSRGVNYGQP (SfH)   (f100;p420)

             cAQGMGGAFGGGQGMGGAVRGGQGMGGA (SfI)   (f82ᵃ;p22)

                                    cGEAEADNADYDEYSDSLDE (SfJ)   (f16;p17)

                                    cGEAEADNADY (SfK)   (f36;p37)

                                      cADNADYDEYSD (SfL)   (f64;p92)

                                          cDEYSDSLDE (SfM)   (f13;p10)

                                          sDEYSDSLDE (SfN)   (f18;p12)

                                          cDEYSDtLDE (SfO)   (f3.8;p3.6)

                                    cGEAEADNADYDEYSDtLDE (SfP)   (f6.5;p2.2)

                        cRGGQGMGGAVGGGQFGAFSPGEAEADNADYDEYSDtLDEGD (SfQ)   (f8.2;p12)


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               ANGVRQNVLNNINANAPGNAGYGGQGGMGA (SfR)   (f8.6;p120)
                           (species specific)
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Figure 2. *Strongylocentrotus franciscanus* bindin sequence and peptides. The sequence is from Minor *et al.* (1991); nomenclature as in Figure 1. "L1-L3" mark copies of the long consensus sequence, "inv rep" marks 10 amino acids translated from a 30-nt stretch that is also present as an inverted repeat in the 5' untranslated region of the *S. franciscanus* bindin mRNA, and "S1-S2" mark copies of the short consensus sequence. Underlined amino acids are those that differ between the bindins of the two species. SfH-SfR indicate the synthesized peptides. The double underlined "t" in peptides SfO, SfP, and SfQ indicates that these peptides were synthesized with a serine altered to threonine. ᵃThis value is from an experiment in which the Hill coefficient n < 1 (see text).

Data from all the inhibition experiments performed are listed in Table 1 (*S. purpuratus* fertilizations) and Table 2 (*S. franciscanus* fertilizations).

The IC$_{50}$ values observed ranged from 2.2 μM up to 740 μM for different peptides, as shown in Tables 1 and 2. The magnitude of this range means that the inhibition cannot be due to any contaminant common to the peptide synthesis or purification. The consistency between different preparations can be seen in the results for peptides SfM and SfN. These peptides were made on different synthesis runs and differ only at the N-terminal amino acid. The values for n and IC$_{50}$ (Tables 1 and 2) are similar for both of these peptides, so the results obtained do not depend on a particular peptide synthesis preparation. Peptide SfQ was further purified by FPLC, and the results obtained after FPLC were the same as before the FPLC purification.

Control Experiments

Several experiments were performed to determine when the peptides acted to inhibit fertilization and to eliminate the possibility that the peptides affected membrane elevation artifactually or poisoned either sperm or eggs.

The Peptides do not Activate Eggs. Because various substances activate eggs and produce membrane elevation irrespective of fertilization, all of the peptides used in these studies were tested for this property. None of the peptides were found to cause elevation of the fertilization membrane when incubated with eggs in the absence of sperm.

The Peptides do not Affect Subsequent Cleavage or Fertilization and do not Inhibit Membrane Elevation While Permitting Cleavage to Occur. The effect of the peptides on the development of fertilized eggs was monitored by observing the eggs through cleavage. Peptides SpC, SfO, and SfR were tested on both *S. purpuratus* and *S. franciscanus* fertilizations. Duplicate treatments were fixed at either 5 min or 2 h after the addition of sperm. At peptide concentrations ranging from the IC$_{50}$ value up to four times the IC$_{50}$ value, the number of eggs that had cleaved at 2 h was the same as the number of eggs that had an elevated fertilization membrane at 5 min. Thus, the peptides did not inhibit or delay the cleavage of the fertilized eggs. There were no observed cases of eggs that had cleaved without an elevated fertilization membrane, indicating that these
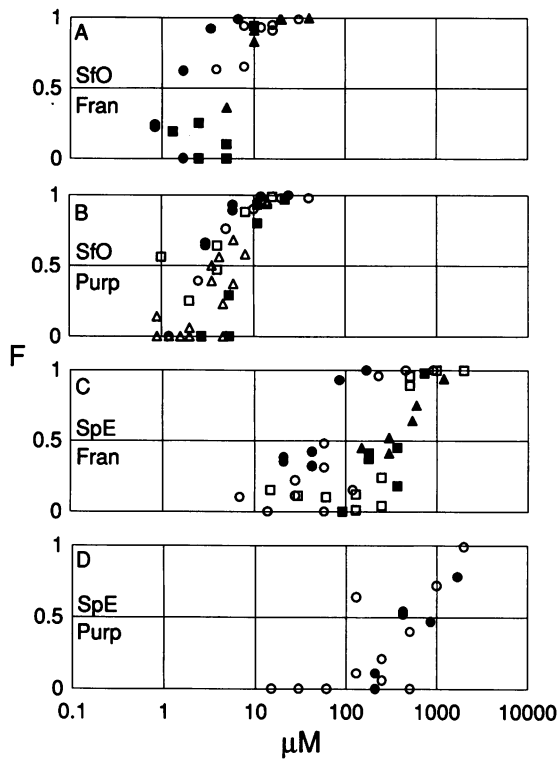
Figure 3. Inhibitory effects of two different bindin-derived peptides on fertilization. The data are graphed as μM peptide concentration (log scale, X-axis) versus fraction of sperm contacts inhibited (F, Y-axis). Different data symbols represent independent experiments done on different days with different batches of eggs and sperm. A–D show measurements done with peptides SpE and SfO on *S. franciscanus* eggs (Fran) or *S. purpuratus* eggs (Purp). Mean IC_{50} values are SfO/Fran, 3.8 μM; SfO/Purp, 3.6 μM; SpE/Fran, 220 μM; and SpE/Purp, 510 μM.

peptides were not causing an apparent inhibition of fertilization, i.e., by merely preventing the elevation of the fertilization envelope. Therefore, membrane elevation is a perfectly reliable index of successful fertilization in the presence of the peptides.

Membrane Elevation also Occurs Normally in the Presence of the Peptides when Induced by Ca Ionophores. The possibility that the peptides could artifactually prevent the elevation of the fertilization envelope per se was addressed additionally, by adding the Ca ionophore A23187 to the peptide-treated eggs. Peptides SpC, SfO, and SfR were tested on both *S. purpuratus* and *S. franciscanus* eggs. At peptide concentrations that were eight times the IC_{50} values, when over 90% of the sperm contacts were inhibited in the duplicate treatments with added sperm, the addition of A23187 caused the elevation of all of the fertilization envelopes. This is a direct demonstration that these peptides do not inhibit the elevation of the fertilization envelope. This experiment confirms that fertilization can be assessed

by observation of membrane elevation as accurately in the presence of the peptides as in normal control eggs. **The Peptides have no Toxic Effects on Either Eggs or Sperm.** The possibility of toxic effects of the peptides on both eggs and sperm was evaluated by washout experiments. The egg washout experiment tested peptides SpF, SfH, SfN, and SfP on *S. purpuratus* eggs. In this experiment, after the 1-h coincubation of eggs with peptide, the MPFSW containing the peptide was removed by aspiration and replaced with fresh MPFSW. After 1 min, the eggs were fertilized as usual. The eggs in this experiment (peptide treated and then washed) fertilized at the same levels as the untreated eggs, which indicates both that the eggs had not been poisoned by the peptides and that the inhibitory effects of the peptides are removed quickly by washing. In the sperm washout experiment, we tested peptides SpB, SpC, SpD, SpE, SfI, and SfP on both *S. purpuratus* and *S. franciscanus* eggs. In this experiment the sperm were diluted into MPFSW containing the peptide. After a 5-min coincubation of sperm and peptide, 40 μl of the sperm-peptide mixture was diluted into 1 ml of MPFSW containing the eggs, and the assay proceeded as in the inhibition assay. In no case was inhibition of fertilization observed when IC_{50} concentrations of peptide were added to the sperm. Thus, at the IC_{50} concentrations, these peptides do not inhibit fertilization by poisoning either the sperm or the egg.

The Peptides Neither Induce nor Interfere with Sperm Activation. Effects of the peptides on the acrosome reaction were monitored by the phenyl bead method of Yamada and Aketa (1988). This method takes advantage of the fact that only acrosome reacted sperm
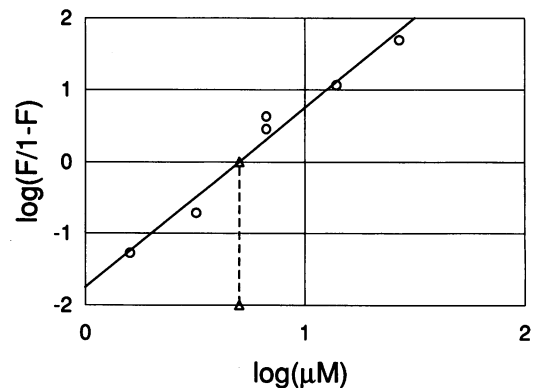

Figure 4. Hill plot of peptide inhibition of fertilization. The data plotted are for a single experiment using peptide SfP to inhibit *S. purpuratus* fertilization. The line is fitted by least-squares linear regression using the Hill equation (Eq. *11*, MATERIALS AND METHODS) and its slope is the Hill coefficient n (in this experiment n = 2.5) The point where fertilization is half inhibited (F = 0.5) occurs when $\log(F/(1 - F)) = 0$. The dotted line indicates the concentration of peptide required for 50% inhibition. In this case, at F = 0.5, IC_{50} = 5 μM.

Table 1. Hill coefficients and 50% inhibitory concentrations for peptides used to inhibit *S. purpuratus* fertilization[a]

Peptide	n	IC$_{50}$[b]
CP2	3.0 ± — (1)	670 ± — (1)
SpE	1.4 ± 0.5 (3)	510 ± 170 (2)
SfH	1.8 ± 0.8 (4)	420 ± 100 (3)
SpB	2.1 ± 2.1 (4)	210 ± 130 (2)
CP1	3.2 ± 0.5 (2)	200 ± 80 (2)
SpD	1.8 ± 0.8 (3)	190 ± 70 (2)
CP4	2.6 ± 1.7 (2)	170 ± 160 (2)
CP5	1.5 ± 0.1 (4)	110 ± 60 (4)
SfL	1.5 ± 0.4 (4)	92 ± 77 (3)
CP6	1.8 ± 0.4 (2)	58 ± 13 (2)
CP3	1.3 ± 0.1 (4)	40 ± 31 (4)
SfK	2.6 ± 0.4 (3)	37 ± 12 (3)
SpG	1.5 ± 0.4 (3)	26 ± 6 (3)
SfI	1.5 ± 0.6 (2)	22 ± 2 (2)
SfJ	2.2 ± 0.4 (3)	17 ± 6 (3)
SpA	1.6 ± 0.6 (4)	16 ± 8 (3)
SpF	0.8 ± 0.2 (2)	16 ± — (1)
SpC	1.6 ± 0.3 (4)	15 ± 6 (4)
SfQ	2.1 ± 0.4 (4)	12 ± 4 (4)
SfN	2.5 ± 0.4 (5)	12 ± 9 (5)
SfM	2.2 ± 0.8 (4)	10 ± 7 (4)
SfO	2.3 ± 0.7 (5)	3.6 ± 1.8 (5)
SfP	1.7 ± 0.5 (5)	2.2 ± 1.6 (5)
SfR	1.2 ± 0.6 (6)	120 ± 40 (3)

[a] Values are means ± SD; number of determinations are in parentheses.

[b] Average (in μM) of experiments when n ≥ 1.

Peptides are arranged from the least inhibitory to the most inhibitory. For the first group of peptides the IC$_{50}$ values are all >20 μM. For the second group the IC$_{50}$ values are <20 μM. Twenty micromoles was chosen as the arbitrary dividing line between "inactive" and "active" peptides because the lowest inhibitory value seen in the control peptides was 40 μM (CP3). The last peptide (SfR) was the only peptide that displayed a statistically significant species difference in IC$_{50}$ values (see also Table 2).

stick to phenyl Sepharose beads. Sperm and phenyl beads were mixed either in the presence or absence of acrosome reaction inducers. When *S. purpuratus* sperm are induced to undergo the acrosome reaction by egg jelly, an average of ~20 sperm adhere per bead perimeter (compared with ~1 in the absence of egg jelly). In the case of *S. franciscanus* sperm, ~30 sperm adhere per bead perimeter. Higher numbers of sperm per bead perimeter are obtained when the acrosome reaction is induced (in the absence of egg jelly) with either A23187 (Vacquier, 1986) or MPFSW containing 30 mM tris(hydroxymethyl)aminomethane (Tris) 9.8 (Kinsey et al., 1980). The effects of peptides SfM, SfO, SfQ, and SfR were tested on both *S. purpuratus* and *S. franciscanus* sperm. In the absence of egg jelly, IC$_{50}$ concentrations of the peptides did not increase the number of sperm per bead perimeter 0.64-2.2 for *S. purpuratus*, 0.5-1.5 for *S. franciscanus*). At 8 times the

IC$_{50}$ concentrations (in the presence of egg jelly), the peptides caused no decrease in the number of sperm per bead perimeter (15-24 for *S. purpuratus*, 26-36 for *S. franciscanus*). These results indicate that these peptides do not either induce or inhibit the acrosome reaction.

Inhibition of Fertilization by Peptides does not Depend on Length of Time they are Coincubated with Eggs. In the inhibition assay, peptide is coincubated with the eggs for 1 h. To determine the minimum coincubation time required for inhibition of fertilization, shorter periods of coincubation ranging from 1 min to 1 h were performed. Peptides SpF, SfH, SfN, and SfP were tested on *S. purpuratus* eggs. For these peptides, the fertilizations in the 1-min coincubation were as inhibited as those in the 1-h coincubation. Taken together, the results of these experiments and the washout experiments indicate that the peptides inhibit fertilization quickly and reversibly.

Direct Measurement of Interference of Sperm Binding to Egg Surface Mediated by Bindin Peptides. Because the bindin peptides indeed inhibit fertilization, without affecting the activation or viability of either sperm or eggs, and because bindin mediates binding of sperm to egg receptor, the peptides must function by blocking sperm binding. This was directly observed by counting the number of sperm adhering to the optical

Table 2. Hill coefficients and 50% inhibitory concentrations for peptides used to inhibit *S. franciscanus* fertilization[a]

Peptide	n	IC$_{50}$
CP2	5.0 ± — (1)	740 ± — (1)
CP1	2.3 ± — (1)	260 ± — (1)
SpE	1.3 ± 0.7 (7)	220 ± 170 (5)
SfH	1.8 ± 1.2 (4)	100 ± 90 (3)
SpB	1.5 ± 0.6 (3)	92 ± 40 (2)
SfL	2.0 ± 1.3 (3)	64 ± 16 (3)
SpD	2.3 ± 0.8 (4)	38 ± 25 (4)
SfK	1.9 ± 0.1 (2)	36 ± 11 (2)
SfI	0.7 ± 0.3 (3)	— ± — (—)
SfN	2.5 ± 0.9 (4)	18 ± 7 (4)
SfJ	2.8 ± 0.8 (4)	16 ± 5 (4)
SpC	1.9 ± 0.5 (3)	13 ± 5 (3)
SfM	3.2 ± 0.8 (5)	13 ± 3 (5)
SpG	3.0 ± 1.1 (5)	12 ± 6 (5)
SpF	2.8 ± 1.1 (2)	12 ± 17 (2)
SfQ	2.6 ± 0.9 (5)	8.2 ± 5.1 (5)
SfP	2.6 ± 2.3 (3)	6.5 ± 4.1 (2)
SpA	3.3 ± 0.9 (3)	5.5 ± 4.8 (3)
SfO	2.5 ± 1.0 (4)	3.8 ± 2.0 (4)
SfR	2.4 ± 0.9 (4)	8.6 ± 5.2 (4)

Note. Values are arranged as in Table 1. The last peptide (SfR) was the only peptide that displayed a statistically significant species difference in its inhibitory concentrations (see also Table 1).

perimeter of eggs at various peptide concentrations. Experiments were carried out with peptides SfO and SfQ. When these were added to eggs immediately before the addition of sperm, the number of sperm that bind to the eggs is reduced, as a monotonic function of peptide concentration. Data from one of these experiments are shown in Figure 5. At the concentrations used, peptides SfO and SfQ sharply reduced the number of sperm-egg contacts, whereas a control peptide (CP2) did not lower the number of sperm bound to the eggs. These experiments were repeated with the gametes of both species, with the same result. Although this experiment indeed provides a direct demonstration that the bindin-derived peptides prevent fertilization by interfering with sperm binding, there are several reasons why this type of experiment was not routinely used. First, this assay requires extremely high nonphysiological sperm concentrations (cf. Pennington, 1985). Second, the number of sperm loaded onto the eggs (even in the control case) changes quickly in a matter of seconds as the first sperm to bind fertilizes the egg and initiates the cortical reaction, which in turn removes adherent sperm from the egg surface (Vacquier *et al.*, 1973; Vacquier and Payne, 1973; Vacquier *et al.*, 1979). For these reasons, the inhibition of fertilization is a far more quantitative and reliable measure of relative peptide effectiveness than is counting of bound sperm.

Bindin Surface Contacts

Peptides were selected that collectively cover all areas of the *S. purpuratus* bindin where there were two or more consecutive amino acid sequence differences with the *S. franciscanus* bindin sequence (Figure 1) and all areas of the *S. franciscanus* bindin sequence where there were three or more differences with the *S. purpuratus* sequence (Figure 2). A total of 24 peptides were tested for their activity as inhibitors of fertilization. Of these, 10 were active inhibitors, defining an "active" peptide arbitrarily as one for which the IC_{50} value is ≤ 20 μM. None of the control peptides inhibited fertilization by this criterion. As Tables 1 and 2 show, only about one-third of the different bindin-derived peptides are active inhibitors of fertilization, if the fact that peptides SfJ-SfQ represent the same region of the bindin sequence is taken into account (see Figure 2).

Peptide SfJ was the first of the *S. franciscanus* bindin peptides that we scored as an active inhibitor, and the size and sequence requirements of the contact defined by this peptide were investigated using other peptides related in sequence. These are shown in Figure 2. Peptides SfK, SfL, SfM, and SfN were synthesized to cover the ends and middle of peptide SfJ. Peptides SfK and SfL were not potent inhibitors of fertilization, but peptides SfM and SfN retained the inhibitory activity of
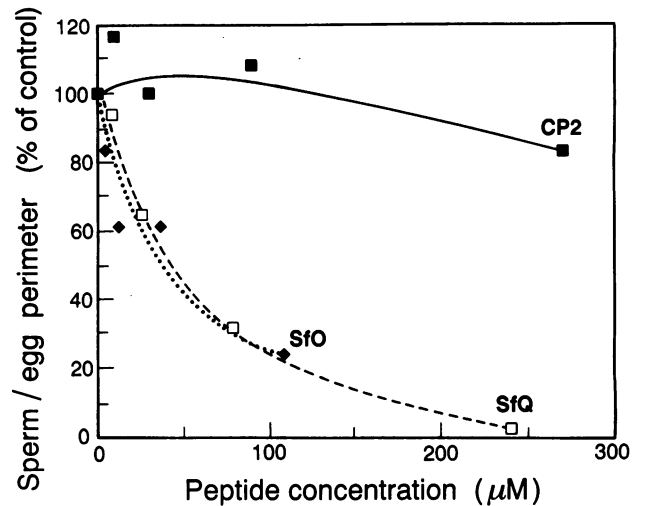

Figure 5. Direct observation that bindin-derived peptides specifically inhibit binding of sperm to egg. The indicated amounts of peptides (CP2, SfO, and SfQ) were added to *S. purpuratus* eggs immediately before mixing with *S. purpuratus* sperm. The data are graphed as μM peptide concentration (X-axis) versus the number of sperm visible per egg optical perimeter (as percent of control, Y-axis). The bindin-derived peptides (SfO and SfQ) inhibited sperm-egg binding, whereas the control peptide (CP2) was ineffective.

peptide SfJ (Table 2). These two peptides correspond to the C-terminus of peptide SfJ and contain only four amino acids that are not on the inactive peptide SfL. These amino acids are SLDE; if the serine is altered to threonine (TLDE), the peptides carrying the alteration (SfO, SfP, and SfQ) have increased inhibitory activity (Figure 2, Table 2). Although this might seem a large effect for a conservative amino acid change, in potassium channels the reciprocal conservative change (threonine to serine) results in alterations of both the ionic conductivity (Yool and Schwarz, 1991) and the tetraethylammonium sensitivity (Yellen *et al.*, 1991) of the channels. In our fertilization assays, peptides carrying the inhibitory S/TLDE site are effective whether they are as small as 10 amino acids (SfM, SfN, and SfO) or as large as 42 amino acids (SfQ).

The peptides selected for study contain several features of the *Strongylocentrotus* bindin sequences that have been defined previously by sequence comparisons (Minor *et al.*, 1991). Peptide SpD contains the polyglycine stretch (Figure 1) and is a poor inhibitor of fertilization. Peptide SfI contains copies of the L repeat (Figure 2) and is ineffective. Peptides SpE, SpF, and SpG all contain copies of the S repeat (Figure 1) and have varying effects on fertilization: SpE is ineffective, SpF is effective, and SpG is borderline. Thus, although some of the amino acids carried on peptide SpF that vary between the S repeats might confer inhibitory activity, these results suggest that the S repeats themselves do not define effective inhibitory regions.

Peptide SpC was synthesized to cover part of the conserved core sequences (Figure 1) and is centered on the sequence LRHLRHHSN. This region was previously identified by DeAngelis and Glabe (1990a) as a sulfated fucan binding site in bindin. DeAngelis and Glabe (1990a) showed that the peptide LRHLRHHSN binds to sulfated fucans, causes egg adhesion, and inhibits fertilization, and both the adhesive and inhibitory properties of LRHLRHHSN require added zinc. However, the concentration of LRHLRHHSN necessary for half maximal inhibition of fertilization is 400 μM (De Angelis and Glabe, 1990a). Peptide SpC shares both the egg adhesive and fertilization inhibitory properties of LRHLRHHSN (Figure 1, Tables 1 and 2). Unlike LRHLRHHSN, peptide SpC does not require added zinc for either its adhesive or inhibitory activities. In addition, SpC is a much more potent inhibitor of fertilization than is LRHLRHHSN, displaying an IC_{50} value of 15 μM. Because the half maximal inhibitory concentration (400 μM) of LRHLRHHSN places it among the least potent of the inhibitory peptides in our study (cf. control peptides CP1, CP2, CP4, and CP5; Table 1), it is likely that the higher fertilization inhibitory activity of peptide SpC is due to the SpC sequence that is not included within LRHLRHHSN. Furthermore, the bindin sequence corresponding to peptide SpC is identical in both species of *Strongylocentrotus*, and SpC inhibits the fertilization of both *Strongylocentrotus* species equally well (Figure 1; Tables 1 and 2).

Species Specificity of Inhibition of Fertilization by Peptide SfR

The only peptide used in this study for which there was a clear and statistically significant difference in IC_{50} val-
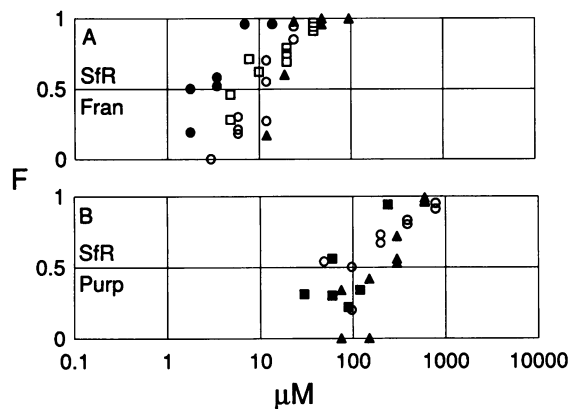

Figure 6. Species-specific inhibition of fertilization by peptide SfR. The format is the same as for Figure 3. Different data symbols represent independent experiments done on different days with different batches of eggs and sperm. (A) Inhibition of *S. franciscanus* fertilization by peptide SfR; mean IC_{50} value is 8.6 μM. (B) Inhibition of *S. purpuratus* fertilization by peptide SfR; mean IC_{50} value is 120 μM.
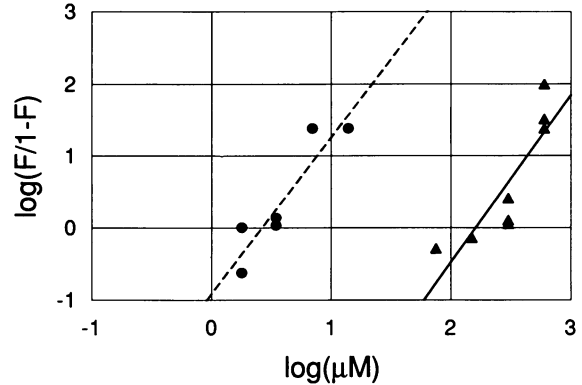

Figure 7. Hill plot of peptide SfR inhibition of fertilization of both *S. purpuratus* and *S. franciscanus*. One individual experiment is shown for each species. The data symbols (●, *S. franciscanus* experiment; ▲, *S. purpuratus* experiment) correspond to the experiments with the same symbols in Figure 6. The fit for the *S. franciscanus* experiment (solid line) yields a Hill coefficient n of 2.2 and a 50% inhibitory value (IC_{50}) of 2.6 μM. For the *S. purpuratus* fit (dotted line) n = 2.3 and IC_{50} = 160 μM.

ues between the two species was SfR, a peptide from a region of the *S. franciscanus* bindin that in sequence is unique to this species (see Figure 2). The inhibitory effects of peptide SfR on both *S. franciscanus* and *S. purpuratus* fertilizations are shown in Figure 6. Figure 6A shows the inhibitory effect of peptide SfR on the fertilization of *S. franciscanus*. Four separate experiments were performed, each using a different male and female. The mean IC_{50} value for these experiments is 8.6 μM (Table 2, last entry). Figure 6B shows the inhibitory effects of peptide SfR on three different *S. purpuratus* fertilizations. The mean inhibitory value for these experiments is 120 μM (Table 1, last line). Representative Hill plots for one experiment from each species are shown in Figure 7. As can be seen in both figures, there is a >10-fold difference in the IC_{50} concentration for this peptide between the two species. This difference between the species indicates a high degree of species specificity in the inhibitory activity of peptide SfR.

DISCUSSION

Comparison to other Peptide Inhibition Studies

The results reported here indicate that small soluble peptides derived from the sperm protein bindin are capable of inhibiting fertilization. Eleven of the 24 peptides tested had IC_{50} values below 20 μM, the most inhibitory being SfP (IC_{50} = 2.2 μM). The IC_{50} concentrations of these peptides are in the range of the most active peptides found to inhibit association in comparable studies on other systems, utilizing peptides of roughly equivalent lengths. These include peptide inhibition of the interaction of HEL(46-61)-peptide with

the MHC protein I-A^k (IC$_{50}$ = 10 μM) (Buus *et al.*, 1987); transducin with anti-transducin monoclonal antibody 4A (IC$_{50}$ = 10 μM) (Hamm *et al.*, 1988); α-bungarotoxin with the acetylcholine receptor (IC$_{50}$ = 17 μM) (Lentz *et al.*, 1988); and transducin with rhodopsin (IC$_{50}$ = 50 μM) (Koenig *et al.*, 1989). The most active peptides from each study are cited here; in all these studies, peptides with much higher IC$_{50}$ values were also tested.

With the exception of the bindin-derived peptide LRHLRHHSN investigated by DeAngelis and Glabe (1990a), we are unaware of other studies using unmodified peptides to inhibit fertilization. The peptide LRHLRHHSN inhibits fertilization 50% at 400 μM and only in the presence of 0.4 μM zinc (DeAngelis and Glabe, 1990a). The modified peptide chymostatin inhibits fertilization at 150–200 μM (Hoshi *et al.*, 1979; Glabe *et al.*, 1981). Roe *et al.* (1988) reported two modified peptides, succinyl-alanyl-alanyl-phenylalanyl-4-aminomethylcoumarin and carbobenzoxy-glycyl-phenylananyl-NH$_2$, which inhibited fertilization 50% at 450 μM and 1370 μM, respectively. Matsumura and Aketa (1991) reported that the modified peptide succinyl-lysyl-lysyl-valinyl-tyrosinyl-metylcoumaryl-7-amide inhibited the acrosome reaction at 800 μM. Hoshi *et al.* (1979) also reported that soybean trypsin inhibitor, leupeptin, and antipapain did not inhibit fertilization but did inhibit the elevation of the fertilization envelope. In this study we demonstrated that peptides SpC, SfO, and SfR do not inhibit the elevation of the fertilization envelope of either species of sea urchin (both by monitoring eggs through cleavage and by using the calcium ionophore A23187). The concentrations of peptides required to inhibit fertilization in all the previous studies cited lie within (or above) the range of the IC$_{50}$ values for the entirely unrelated control peptides we used (see Tables 1 and 2) and all are far higher than the 20-μM concentration limit we set as the definition of an active inhibitory peptide.

Cooperative Peptide Inhibition of Fertilization

A general property of the inhibitory effects of these peptides is a steep dose response, i.e., the inhibition changes from no inhibition to complete inhibition over a narrow interval of concentration (e.g., Figures 3 and 6). The steepness of this dose response is reflected in the Hill coefficient, n, which is the slope of the line fit to the Hill equation (Eq. 11, Figures 4 and 7). The average of the Hill coefficients for all the peptides used in this study is 2.1 ± 0.8 (mean ± SD); the average of the peptides active on *S. franciscanus* eggs is 2.7 ± 0.4. This Hill coefficient indicates that the concerted action of two to three peptides (on average) is necessary to inhibit fertilization. An analogous situation occurs in the peptide inhibition of rhodopsin-transducin association (Koenig *et al.*, 1989). The rhodopsin-derived inhibitory peptides

also demonstrate a steep dose response, with Hill coefficients of two to three, because they interfere with a cooperative binding process.

Bindin is packaged as an insoluble granule of protein inside the sperm acrosome. When the sperm contacts the egg, the exocytosis of the acrosome exposes bindin on its surface. The bindin granule remains associated with the sperm acrosomal membrane and forms a large contact with the surface of the egg. The size of this contact varies but can be estimated to be $\sim$0.025 μm^2 from electron micrographs of sperm-egg contacts in which bindin has been stained with immunoperoxidase (Moy and Vacquier, 1979). Given this area and the molecular weight of bindin, there could be as many as 10^3 bindin monomers in the contact region between the sperm and the egg, though only a small fraction of these might be engaged productively with the bindin receptor on the egg surface. The intact bindin receptor is a very large glycoprotein complex (Glabe and Vacquier, 1978; Rossignol *et al.*, 1984b). Digestion of the intact receptor with lysylendoproteinase C releases a 70-kDa fragment that retains the ability to bind species specifically to bindin (Foltz and Lennarz, 1990). The specificity of the peptide inhibitions reported here implies that the peptides are occupying portions of the recognition sites on these receptors. Thus, the average Hill coefficient of 2.7 can be interpreted as indicating that if at each sperm binding site two to three receptor target sites are occupied by peptides, this will prevent sperm from binding productively. These data cannot be used to determine how many bindin-receptor interactions are actually involved in the formation of a mature sperm-egg contact, and from the size of the contact area this could be a large number.

Species Specificity of the Bindin–Receptor Interaction

Earlier sequence comparisons between the mature bindins of *S. franciscanus* and *S. purpuratus* revealed that the middle third of the protein is conserved between the two species, whereas the N- and C-terminal thirds display both point changes and variations in the numbers of direct repeats of 7–10 amino acid long sequence elements (Minor *et al.*, 1991). These changes are marked as underlined amino acid residues in the bindin sequences shown in Figures 1 and 2. One explanation of how these changes in bindin could affect species specificity is to assume that at least certain of the variable sequences encode species-specific binding domains on the surface of the bindin molecule and that these sequences are matched by complementary species-specific target sites on the receptor. An alternative explanation of the cause of species specificity, the "supramolecular theory" (Glabe, 1978, 1979), postulates that specificity could be due to the same binding site being presented

in different, species-specific, ordered arrays on the surfaces of the sperm of the two species. In the supramolecular model, a randomly packed field of bindin-receptor contacts would have insufficient strength to maintain sperm binding. A "threshold" value for binding could only occur between species-compatible arrays of ordered monomers in the bindin granule and ordered receptors in the vitelline envelope.

Peptide SfR is derived from a unique region of the *S. franciscanus* bindin, and this peptide species specifically inhibits *S. franciscanus* fertilization. This fact directly supports the notion that the species specificity of the bindin-receptor interaction is due to a sequence specific recognition process. This result would not be expected if species specificity were due to different ordering of arrays presenting the same binding site, as predicted by the supramolecular theory.

Five different areas of the bindins were identified that could be involved in direct surface contacts between bindin and its receptor. These regions are represented by the peptides SfR, SpC, SpA, SpF, and SfM. Peptide SfR inhibits fertilization species specifically, whereas the other four peptides inhibit the fertilization of both species equally well. The lack of a species difference in the activity of the peptide SpC is the easiest to understand, because the amino acid sequence of peptide SpC is identical in both species of bindin. Therefore, peptide SpC represents an area of bindin involved in a contact (probably to sulfated fucans) (DeAngelis and Glabe, 1988, 1990a,b) that is conserved between the two species. Peptides SpA, SpF, and SfM also might be involved in conserved contacts, despite the differences between the two bindins in these areas. Thus, some amino acids are conserved between the bindins of these two species in these areas, and the ability of the SpA, SpF, and SfM to inhibit the fertilization in both species could be explained if it is the conserved amino acids that form the important contacts with a conserved receptor structure. It is also possible that the bindin regions represented by these peptides have indeed changed functionally between the two species, but the corresponding target site on the receptor has not changed. Such "vestigial" characters, if present in the receptor, could make it possible for a peptide derived from a unique area of the bindin of a given species to inhibit the fertilization of both species. In any case, the data indicate that there are five subregions of the bindins that are involved in the sperm-egg contact, but only one of these regions, that included on the 30 amino acid long peptide SfR, seems to constitute a species-specific binding domain. These results suggest a specific series of experiments using recombinant bindin proteins, in which the active regions have been altered by in vitro mutagenesis, and such experiments are now in progress in our laboratory.

There are many sequence differences in the region homologous to SfR in the sea urchin bindins that have

been sequenced, as shown in Figure 8. The most notable change between the *Strongylocentrotus* species is a three amino acid insertion in the *S. franciscanus* bindin sequence, relative to the homologous area of the *S. purpuratus* bindin. Curiously, the sequence encoding these three amino acids is included in a 30 nt long stretch of DNA also present as an inverted repeat (29/30 nt match) in the 5' untranslated region of the *S. franciscanus* bindin mRNA (Minor et al., 1991).

Evolutionary Implications

Many marine invertebrates utilize external fertilization and planktonic larvae. The species specificity of gamete interaction is likely to play a major role in establishing the reproductive isolation necessary for speciation in such organisms, as in many cases congeneric species with overlapping breeding seasons are found within easy range of each other's broadcast gametes. This is the case, for example, with the sea urchin species studied here, *S. purpuratus* and *S. franciscanus* (Boolootian, 1966; see Chapter 1 in Minor, 1992). The results we report imply that changes in the species specificity of gamete interaction could result from a small change in the primary sequence of the sperm recognition protein. Peptide SfR is derived from a unique area of the *S. franciscanus* bindin and specifically inhibits *S. franciscanus* fertilization. The peptide covering the homologous region of the *S. purpuratus* bindin (SpD) is an ineffective inhibitor of fertilization of both species. These results can be interpreted to indicate a gain of a unique interaction region on one molecule (e.g., the bindin of *S. franciscanus*) that has been accompanied by a compensatory change in its partner (i.e., the *S. franciscanus* receptor), thus creating a novel and specific sperm binding site. Further understanding of how changes in sperm-egg recognition evolved will require studies of the structures of both

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ANGVRQNVLNNINANAPGNAGYGGQGGMGA   Peptide SfR

ANGVRQNVLNNINANAPGNAGYGGQGGMGA   S. franciscanus

AtGaqQgVLNg---NAPGqAGfGGgGGgGA   S. purpuratus
                      t
ANGVReNVLNN1--NAPGqgGYGGQGGMrg   L. variegatus

ANGVRdNVLNN1N-egPG-AGavagaaMaA   A. punctulata
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Figure 8. Sequences of the species-specific inhibiting peptide SfR and the homologous regions of bindins from other species. Sequence data are from the following sources: *Strongylocentrotus franciscanus*, Minor et al. (1991); *S. purpuratus*, Gao et al. (1986); *Lytechinus variegatus*, Minor et al. (1991); and *Arbacia punctulata*, Glabe and Clark (1991). Amino acids deleted relative to the *S. franciscanus* bindin are shown as dashes; mismatched amino acids are shown in lower case. A threonine insertion in the *Lytechinus* sequence is shown as a "t" over the glycine that it follows. The underlined sequence in the *S. franciscanus* bindin sequence indicates 10 amino acids encoded by a DNA sequence in the coding region of the *S. franciscanus* bindin mRNA that is also contained in the 5' untranslated region as an inverted repeat (Minor et al., 1991).

bindin and the receptor responsible for the specific recognition. The recent demonstration that a soluble receptor fragment retains the ability to species-specifically recognize bindin (Foltz and Lennarz, 1990) enormously improves prospects for such structural studies. Studies of variant functional domains and their distribution in populations may elucidate the structures responsible for the specificity of sperm adhesion and provide information on how these changes might have been involved in gene pool separation, thus resulting in the speciation of these sympatric marine organisms.

ACKNOWLEDGMENTS

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