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The hsp70 protein is involved in the acquisition of gamete self-sterility in the ascidian *Ciona intestinalis*

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SUMMARY

In the hermaphrodite ascidian *Ciona intestinalis*, gamete self-incompatibility is a mechanism that prevents self-fertilization and is based on the ability of the oocyte vitelline coat to distinguish and accept only heterologous spermatozoa. The onset of self-sterility occurs during oogenesis and involves or is controlled by the follicle cells. Gamete self-nonself discrimination, a process that can be likened to an immune recognition event, represents a useful model with which to study the evolution of self-nonself recognition. Hsp70 genes, which belong to the major histocompatibility complex (MHC) class III, are supposedly ancestors of the MHC class I and II genes, and chaperonins are known to be involved in antigen processing and presentation. We have isolated and characterized an *hsp70* gene (*Cihsp70*) that is constitutively expressed during oogenesis in the follicle cells of previtellogenic and vitellogenic oocytes. Using a polyclonal antibody against Cihsp70 protein, we demonstrate that the expression of *Cihsp70* is required for the switch from self-fertility to self-sterility. The functional involvement of *Cihsp70* in gamete self-nonself recognition provides evidence for an ancestral MHC-like system in protochordates.

Key words: *hsp70* gene, Self-nonself discrimination, Oogenesis, Ascidian

INTRODUCTION

All jawed vertebrate species have major histocompatibility complex (MHC) genes and the capacity to respond to MHC-disparate antigens, but the extent to which invertebrate metazoans exploit the MHC and adaptive immunity is obscure (Scofield et al., 1982a). In invertebrates, self-nonself recognition serves to maintain structural integrity and to withstand pathogens (Cooper et al., 1992). Protochordates seem to be a critical link in the phylogeny of the vertebrate immune system. In fact, they possess a hemocyte-mediated allograft rejection mechanism that is based on the rapid recognition of polymorphic surface proteins (Fuke and Nakamura, 1985). This suggests that allogeneic recognition mechanisms and non-specific defence systems are non-interactive and independent processes. Therefore, it is plausible that protochordates have evolved cell-mediated immunity, albeit with a limited 'T-cell receptor (TCR)-MHC' repertoire (Cooper et al., 1992). Gamete self-incompatibility, a mechanism that prevents self-fertilization in self-sterile hermaphrodite ascidians, could be a peculiar form of allorecognition out of an immune context.

Interest in this issue dates back to the work of T. H. Morgan (1923) who observed that the American form of the hermaphrodite self-sterile ascidian *Ciona intestinalis* was 'rarely or never self-fertile' depending on the batch and where the animals were collected. He also provided the first evidence that the control of self-sterility was associated with the egg envelopes and that self-sterility was genetically governed (Morgan, 1923). More recently the presence of self-fertile animals in the wild populations was confirmed, and found to be related to environmental conditions (Kawamura et al., 1987); subsequent studies clarified the role of the egg envelopes in self-nonself recognition. As in the vast majority of animals studied so far, species-specific binding between the egg and the spermatozoon occurs on the vitelline coat (VC), a continuous extracellular layer that surrounds the egg (Rosati and De Santis, 1978; De Santis et al., 1980). Furthermore, in *Ciona intestinalis* the VC also controls gamete self-discrimination. In fact, spermatozoa do not bind to the VC of eggs from the same individual, thus preventing self-fertilization (Rosati and De Santis, 1978). Self-discrimination is established during oogenesis, a process that can be monitored in vitro in isolated ovarian oocytes (De Santis et al., 1991). After germinal vesicle breakdown (GVBD), vitellogenic oocytes are viable to both self and nonself spermatozoa and acquire self-sterility only when fully mature. Products of the overlying follicle cells contribute to or control the onset of self-sterility as demonstrated by the finding that ovarian oocytes deprived of the follicle cells never become self-sterile (De Santis et al., 1991). Follicle cells exert this effect even when detached from the VC and co-cultured with maturing oocytes. The phenomenon is strictly individual-

specific because it occurs only with oocytes and follicle cells from the same animal (Pinto et al., 1995).

Given the invariable success of fertilization between heterologous gametes, the self-sterility barrier is probably regulated by a highly polymorphic system.

In the colonial ascidian *Botryllus* colony fusibility is controlled by a single Mendelian locus at which a large number of alleles segregate in natural populations. This polymorphism is maintained by gamete self-incompatibility systems linked or identical to the fusibility genes (Oka,1970; Scofield et al.,1982b).

Since *Botryllus* fusibility genes bear a strong family resemblance to loci of MHC it has been proposed that they represent primitive MHC genes that are involved in somatic and gamete self-recognition in protochordates (Scofield et al., 1982a).

This prompted us to investigate whether MHC-related molecules are involved in the process of gamete self-recognition in *C. intestinalis*. Attempts, in our laboratory, to detect MHC homologues using nucleic acid hybridization and specific amplification by the polymerase chain reaction (PCR) in *C. intestinalis* as well as in *Botryllus* (Fagan and Weissman, 1996) have been unsuccessful. Hence, a different approach based on the research of genes evolutionarily related to MHC has been undertaken.

Genes encoding hsp70 are present in the MHC class III in a vast range of organisms from humans to *Xenopus*. It is the oldest protein known to possess a peptide binding domain (reviewed by Salter-Cid and Flajnik, 1995), thus an hsp70 molecule is a likely candidate for an ancestral MHC molecule. Recent data obtained in the mouse (Kasahara et al., 1996) indicate that *hsp70* belongs to an ancestral syntenic group of genes that could represent a 'primordial MHC' which is strong evidence that hsp70 was involved in self-nonself discrimination early in evolution. Hence, we studied *hsp70* genes in the *Ciona intestinalis* genome and the function of these genes in gamete self-discrimination.

MATERIALS AND METHODS

Genomic library screening

A genomic DNA library derived from *Ciona intestinalis* sperm was constructed using the Stratagene (La Jolla, CA) lambda-GEM-11 vector according to the manufacturer's protocol. Approximately one million plaques were screened using a ^{32}P-labeled 2.2 kb *Xba*I fragment containing the coding sequence of an *hsp70 Drosophila* gene prepared from the 132E3 clone (Morgan et al., 1979). Hybridization was performed in 50% formamide, 5× SSC, 50 mM sodium phosphate pH 7.2, 5× Denhardt's solution, 0.1% SDS and 0.5 mg/ml tRNA at 37°C for 16-20 hours. Washing was performed at high stringency: 15 minutes at 50°C in 1× SSC with 0.1% SDS and 30 minutes at 65°C in 1× SSC with 0.1% SDS. The analysis of sequences was performed using GenBank nucleic acid sequence database (release 89.0) and the Swiss-Prot amino acid sequence database (release 31.0).

RNA isolation, northern blot and RACE analysis

C. intestinalis ovaries were dissected from the animal and immediately frozen in liquid nitrogen. RNA was extracted with guanidinium thiocyanate followed by centrifugation in cesium chloride solutions. Poly(A)$^+$ RNAs were purified by affinity chromatography on oligo(dT)-cellulose. To determine sequences at the 5′- and 3′-ends of the *Cihsp70* mRNA, rapid amplification of cDNA ends (RACE) assays were carried out using the 5′ and 3′ RACE system (GIBCO BRL). To amplify the 5′ end, the oligo 5′-TTTCCTCGGGTGAAAACGTCTTCACGTCGC-3′ was used for first strand cDNA synthesis of poly(A)$^+$ RNA. After tailing, the purified single strand cDNA was amplified by the PCR using the anchor primer and the nested gene-specific oligo (5′-CAAAAATTGAATTTTCGGGATTTCTGGCAA-3′). The PCR product that hybridized with an oligo (5′-CACCAAGCTATGTCGCATTCAACG-3′) located at the 5′-end of the nested oligo, was subcloned and sequenced. To amplify the 3′-end, an oligo(dT) containing adapter primer was used for first strand cDNA synthesis of poly(A)$^+$ RNA. The first round of cDNA amplification was performed using abridged universal amplification primer (AUAP) and a gene-specific primer (5′ AGTCACACAAATTTGTGACGACACGCA-3′). A second nested gene-specific primer (5′-CAAGATTCACGCATCGGCTGGAGGGCA-3′) was used in a second amplification reaction in conjunction with AUAP to increase the specificity of the procedure. The prominent band on an agarose gel was subcloned and sequenced. These data show that the size of *Cihsp70* mRNA was 2.5 kb with a 3′ UTR of 600 bp.

Poly(A)$^+$ RNA from ovary was electrophoresed on 1.2% agarose/formaldehyde gels and transferred to nitrocellulose. Northern blots were hybridized for 16-20 hours at 65°C in 5× SSC, 5× Denhardt's solution, 5 mM EDTA pH 7.5, 50 mM sodium phosphate, pH 7.2, 0.5% SDS and 0.5 mg/ml tRNA containing ^{32}P-labeled probe A corresponding to part of the ORF and part of the 3′ untranslated region (UTR) spanning positions 1280-2060, or ^{32}P-labeled probe B corresponding to the 3′ UTR of the gene spanning positions 1920-2470. Washing was for 30 minutes at 65°C in 0.2× SSC and 0.1% SDS. In both cases the size of the hybridizing mRNA was 2.5 kb corresponding to that determined by 5′ and 3′ RACE analysis.

Antibody

A polyclonal antibody, which we called V15, was generated by Primm (Milano) against two peptide sequences of the Cihsp70 protein. The two peptides (KNAAEEAGEKLSSA, CDDTQKWLDGNSLAE), located at the COOH-terminus of the protein (see also Fig. 1), the most variable region, were chosen by the Peptidesort program. Experiments were also run using a commercial monoclonal anti-hsp70 antibody (H5147; Sigma).

Immunohistochemistry and in situ hybridization

Ovaries were fixed in 4% paraformaldehyde in 75% sea water at 4°C. Samples were dehydrated, embedded in paraffin, sectioned at 7.5 μm and processed for in situ hybridization (Simeone et al., 1995) and immunohistochemistry. In situ hybridization was carried out with two antisense cRNAs: probe A (see section 'RNA isolation, Northern blot and RACE analysis') and probe C, corresponding to the entire 5′ untranslated region spanning positions –230 to +1. The antisense cRNAs were labeled with ^{35}S-dUTP using the Gemini Riboprobe system (Promega) and purified over a Sephadex G50 column. In situ hybridizations were carried out with 3×10^4 cpm/μl at 55°C. Control experiments were run using the corresponding sense cRNAs.

For immunostaining, sections were washed in TBS, pH 8.8, and saturated with 2% BSA in the same buffer. The primary antibody was

Fig. 1. Genomic DNA sequence of the *C. intestinalis hsp70* gene and the predicted amino acid sequence. A canonical polyadenylation signal (AATAAA) is located 546 base pairs of the stop codon (indicated by capital letters). A potential binding site for heat shock elements (GAANNTTC) is located 172 base pairs 5′ of the start codon (underlined). The translation start site is designated +1. hsp70 family signature sequences are indicated by dotted lines. The three amino terminal signatures are characteristic of hsp70 proteins in general and the three carboxy-terminal signatures are characteristic of eucaryotic non-organellar hsp70 proteins in particular (Rensing and Maier, 1994; Gupta et al., 1994). The two amino acid stretches (double underlined) represent peptides used for production of the polyclonal antibody (V15). The EMBL/GenBank accession number is Y11513.

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-230    cgcgcaatttcgtgttctaaaaataggtggcgcgcatcaacgcttctcgaatatgcgcgaaggttctccattcatcctcg

-150    tgcgtatataatgagacagctcacagcagacggcattctatctagaaaaacgaagcgaaacttaaagaaagaaaacttct
                                                                                    +1
-70     taaagaagatataatattattgtaaagtttattgttacaatactatatagatattcagaagattacaagaATGTCAACAG
                                                                                 M  S  T  A

11      CAGTAGGAATCGATCTGGGAACAACGTATTCATGCGTTGGCGTCTTCCGCCATGGCAAAGTCGAGATTATTGCCAACGAC
          V  G  I  D  L  G  T  T  Y  S  C  V  G  V  F  R  H  G  K  V  E  I  I  A  N  D

91      CAAGGTAATAGAACTACACCAAGCTATGTCGCATTCAACGAAACAGAAAGGTTGATCGGTGATGGGGCCAAAGACCAAGT
        Q  G  N  R  T  T  P  S  Y  V  A  F  N  E  T  E  R  L  I  G  D  G  A  K  D  Q  V

171     TGCCAGAAATCCCGAAAATTCAATTTTTGACGCAAAGAGATTGATTGGAAGAAATTACAACGATCCAGCTGTACAGAAGG
          A  R  N  P  E  N  S  I  F  D  A  K  R  L  I  G  R  N  Y  N  D  P  A  V  Q  K  D

251     ATAAAGAACACTGGCCTTTCAAGGTAGTAAATAAGAATGGAAAACCATTTCTTCAAGCGGAATATCAAGGCGACGTGAAG
          K  E  H  W  P  F  K  V  V  N  K  N  G  K  P  F  L  Q  A  E  Y  Q  G  D  V  K

331     ACGTTTTCACCCGAGGAAATCAGTGCAATGGTTCTTACTAAGATGAAGGACACTGCGGAAGCATACCTCGGTGAAAATGT
        T  F  S  P  E  E  I  S  A  M  V  L  T  K  M  K  D  T  A  E  A  Y  L  G  E  N  V

411     AAAGGACGCGGTTATAACAGTACCTGCTTACTTCAACGACTCACAACGACAAGCAACAAAAGACGCTGGCATCATCGCAG
          K  D  A  V  I  T  V  P  A  Y  F  N  D  S  Q  R  Q  A  T  K  D  A  G  I  I  A  G

491     GACTGAACGTACTCAGAGTGATAAACGAACCCACAGCAGCGGCATTGGCGTACGGACTCGATAAGAACTTGGTTGGCGAG
          L  N  V  L  R  V  I  N  E  P  T  A  A  A  L  A  Y  G  L  D  K  N  L  V  G  E

571     AAAAAGGTTTTAATTTTCGATCTTGGCGGCGGAACCTTCGATGTCTCTGTGCTCACCATCGACGAAGGATCAATTTTTGA
        K  K  V  L  I  F  D  L  G  G  G  T  F  D  V  S  V  L  T  I  D  E  G  S  I  F  E

651     AGTTCTTTCAACTGCTGGGGACACCCATCTTGGCGGAGAGGACTTCGATAACCGAATGGTGAAGCATTTCACTGAAGAGT
          V  L  S  T  A  G  D  T  H  L  G  G  E  D  F  D  N  R  M  V  K  H  F  T  E  E  F

731     TCAAGCGAAAACACAAAAAGGACATCTCAAAGAGTAACAGGGCAATTAGAAGATTACGGACGGCCTGTGAGCGAGCTAAG
          K  R  K  H  K  K  D  I  S  K  S  N  R  A  I  R  R  L  R  T  A  C  E  R  A  K

811     CGCGTCCTCTCAACCTCGACAGAAGCTGCTGTCGAGTTGGATTCTCTATACGAAGGAATTGATTTCTACAGTAAAATCTC
        R  V  L  S  T  S  T  E  A  A  V  E  L  D  S  L  Y  E  G  I  D  F  Y  S  K  I  S

891     AAGAGCAAGATTCGAAGAACTGTGCTCCGATCTCTTCCGTTCCTGTCTTGACCCTGTCGAAAAAGCTCTAAGAGATGCTA
          R  A  R  F  E  E  L  C  S  D  L  F  R  S  C  L  D  P  V  E  K  A  L  R  D  A  K

971     AGCTAGATAAAACGAAGATCGACGAAGTTGTTCTTGTCGGAGGATCAACCAGGATCCCGAGGGTGCAAAACTTACTCAGT
          L  D  K  T  K  I  D  E  V  V  L  V  G  G  S  T  R  I  P  R  V  Q  N  L  L  S

1051    GATTTCTTTAACGGAAAAAATCTTAACAAATCAATCAACCCCGACGAAGCAGTTGCCTACGGTGCTGCGGTGCAGGCGGC
        D  F  F  N  G  K  N  L  N  K  S  I  N  P  D  E  A  V  A  Y  G  A  A  V  Q  A  A

1131    AGTGCTTACAGGCAACTCCGGCGTGAAAGATGTGTTGTTGGTCGACGTTGCACCTCTATCGTTGGGAATTGAGACAGCCG
          V  L  T  G  N  S  G  V  K  D  V  L  L  V  D  V  A  P  L  S  L  G  I  E  T  A  G

1211    GCGAGATGATGACAAAGCTGGTGGAACGAAACACAAGAATCCCACACAATACTTCACAGGTGTTCACCACGTACGCTGAT
          E  M  M  T  K  L  V  E  R  N  T  R  I  P  H  N  T  S  Q  V  F  T  T  Y  A  D

1291    AATCAACCTGCCGTCACAATTCAAGTTTACGAAGGCGAACGAGCACAGACGAAACATAACAATCTGCTCGGAACATTTAA
          N  Q  P  A  V  T  I  Q  V  Y  E  G  E  R  A  Q  T  K  H  N  N  L  L  G  T  F  N

1371    CCTCACTGGAATAGCACCAGCACCAAGAGGTGTGCCAAAGATCAAAGTTTCGTTTGACATCGACGCAAATGGAATCTTGC
          L  T  G  I  A  P  A  P  R  G  V  P  K  I  K  V  S  F  D  I  D  A  N  G  I  L  Q

1451    AAGTCTCCGCCAAAGACGAAAGCACGGGAAAGGCGAACCAGATCACGATCAACAAAGGCCGCTTGTCGAAGGCAGATATA
          V  S  A  K  D  E  S  T  G  K  A  N  Q  I  T  I  N  K  G  R  L  S  K  A  D  I

1531    GAAACTATGCTGGCTGATGCTGAGAAATACAAAGAAGAAGACAATAAACAGATGGAAAGACACAAGGCCAAAAACGAACT
        E  T  M  L  A  D  A  E  K  Y  K  E  E  D  N  K  Q  M  E  R  H  K  A  K  N  E  L

1611    GGAGTCCTACATGTACGGCTGCAAAAATGCTGCCGAAGAGGCCGGTGAAAAATTGTCTTCTGCGGATAAAGATCAAGTCA
          E  S  Y  M  Y  G  C  K  N  A  A  E  E  A  G  E  K  L  S  S  A  D  K  D  Q  V  T

1691    CACAAATTTGTGACGACACGCAGAAATGGCTGGATGGAAATTCTCTTGCAGAAAAGGAAGAGTTTGAAGAGCGACTAAAT
          Q  I  C  D  D  T  Q  K  W  L  D  G  N  S  L  A  E  K  E  E  F  E  E  R  L  N

1771    GAACTAAAAAAGGTTTGCTCGCCCTTAATGAGCAAGATTCACGCATCGGCTGGAGGGCATCAGCAGAATGGCCAAGGAAA
        E  L  K  K  V  C  S  P  L  M  S  K  I  H  A  S  A  G  G  H  Q  Q  N  G  Q  G  K

1851  ACCCGACGGCCCAGTGATCGACGAAATGGACtagatggcttaatctctttttttctattctactcaacttttgtttgtgtaca
        P  D  G  P  V  I  D  E  M  D

1931    tattcacacatttaactcagatccaaccgttcactatttttttctattctacttgactttgtttgtatatactcatatata
2011    aaatttctgtcttactattgaaacttctttatcatgcaatatgtttcctgtagtgttcgtgtatttttcaaatacattatt
2091    taccacaaacatttaatgctttttgaatagccatgacaagatgaaaaacttaggaagaaaggcaccaaaatatttgtatg
2171    actaaaacaaaacagttgttatgtaaaagtacataaaatatttaatgaaagacagaattagatttaaattccagcagatt
2251    ttataaaatttgttacacacatcgcttggtttaataagtatgaaacaaaacggtcttcttcttgcctcttaaggattcgt
2331    tcatgtttagactcatccccaggtattgaggcaagtgtgtcggcataagttttaaaacagtccgaaataggcttggattg
2411    cctcaaaatcgcgtcgacaaataaaaatttcttcaacgaaatctaaaaagatctcaagtaaagtaaagcatttgaaccaa
2491    ccacaaaaggggtctaatgagcgtaacgcagtgctatgcg
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used at a dilution 1/100 in TBS, pH 8.8, for 2 hours at room temperature. Sections were subsequently washed and then exposed to biotinylated anti-rabbit IgG (Sigma). The bound antibody was visualized with FITC-conjugated avidin (Sigma). Controls were run by incubating the sections with preimmune serum normalized with the immune serum by protein content.

Immunoelectron microscopy

Fragments of ovary were fixed in 0.1% glutaraldehyde, 4% paraformaldehyde in 75% Millipore-filtered sea water for 12 hours. Following dehydration, specimens were embedded in LR White Resin (Polysciences Europe GmbH) for 24 hours at 50°C. Thin sections were cut and mounted on 300 mesh nickel or gold grids. Following incubation in 20 mM Tris-HCl buffer, pH 8.8, 137 mM NaCl, containing 1% BSA, samples were exposed to V15 (1:100) for 2 hours at room temperature. Sections were rinsed and incubated with either 10 or 20 nm colloidal gold-labeled Protein A (Sigma) (1:50) for 1 hour. After washing, specimens were stained with uranyl acetate and lead citrate. Control sections were incubated either with preimmune serum or with no primary antibody.

Protein extraction and immunoblotting

Ciona ovaries and muscle were solubilized in lysis buffer (50 mM Tris-HCl, pH 6.8, 150 mM NaCl, 1% NP40) at a 1:5 ratio (v/v) together with a mix of protease inhibitors (Protease Inhibitor Cocktail tablets, Boehringer Mannheim) and homogenized. After 1 hour of incubation at 4°C, samples were centrifuged for 10 minutes at 10,000 ***g*** and the protein content of the supernatant was determined. Aliquots of the samples corresponding to 40 μg of proteins resuspended in Laemmli (1970) sample buffer containing 100 mM DTT, were separated by 8.5% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) and treated with antibody V15 (1:150). All the immunostaining steps were carried out in 20 mM Tris-HCl buffer, pH 8.8, 137 mM NaCl, 0.1% Tween-20 and 5% Non-Fat Dry Milk (BioRad) and detection was performed by ECL (Amersham). For heat shock experiments, animals were incubated at 16° and 24°C respectively for 1 hour. Muscle was then dissected and processed as described above. In vitro protein synthesis was carried out by using the TnT Coupled Reticulocyte Lysate System kit (Promega).

Biological assay

Self-fertile ovarian oocytes were incubated with antibody V15 (1/100 dilution) or a commercial monoclonal anti-hsp70 antibody (1/100 dilution) for 3 hours, which was sufficient time for control oocytes to become self-sterile. In a typical experiment, about 20 oocytes that had undergone GVBD were resuspended in 100 μl of Millipore-filtered sea water. Oocytes were inseminated either immediately after dissection from the gonad or after a 3-hour incubation with 10^5 autologous spermatozoa resuspended in 5 μl of Millipore-filtered sea water. The percentage fertilization was determined by counting the oocytes that underwent the first cleavage. To verify the viability of the oocytes throughout the experiment, cross fertilization controls were run in parallel. In the experiments performed to examine the effect of the antibody, after a 3-hour incubation oocytes were transferred to fresh sea water and autologous spermatozoa were added.

In another series of experiments, follicle cells were separated from oocytes by shaking, as previously described (Pinto et al., 1995). Follicle cell-free ocytes were incubated in small dishes whose bottom consisted of a dialysis membrane (10,000 molecular weight cut off). The dishes were suspended in the wells of Multiwell Tissue Culture Plates that contained the detached follicle cells suspended in sea water. In control experiments spermatozoa were added either after dissection or after a 3-hour incubation. To verify the specific target of the antibody, V15 was added either to the follicle cells or to the oocytes at a dilution of 1/100. Three hours later the oocytes were transferred to fresh sea water and inseminated with autologous spermatozoa.

RESULTS

Isolation and characterization of *C. intestinalis hsp70* gene

We screened a genomic library derived from *C. intestinalis* sperm DNA with a genomic *hsp70* probe from *Drosophila melanogaster* (Morgan et al., 1979). Ten positive lambda clones were isolated and single or double restriction enzyme digestions were performed to obtain the restriction maps. Fragments of two clones that hybridized with the *Drosophila hsp70* probe were subcloned and sequenced. Sequencing resulted in the identification of a single gene (Fig. 1) that displayed polymorphism at the 3′ UTR region. This gene, which we call *Cihsp70*, is intronless and has a single open reading frame of 1884 bp (including the stop codon TAG) encoding a protein of 627 amino acids. A canonical polyadenylation signal (AATAAA) is located 546 base pairs of the stop codon. A potential binding site for heat shock elements (GAANNTTC) (Rensing and Maier, 1994; Gupta et al., 1994) is located 172 base pairs 5′ of the start codon. It displays a sequence identity of about 70% with *Botryllus hsp70* genes (Fagan and Weissman, 1996) and with other inducible members of the *hsp70* gene family.

Northern blot analysis and in situ hybridization

In order to determine whether *Cihsp70* is involved in oocyte maturation, we investigated the expression of this gene in the ovary where all stages of maturation of the oocyte and the accompanying cells are represented.

Northern blot analysis shows that the gene is expressed constitutively in the ovary, while it is turned off in mature

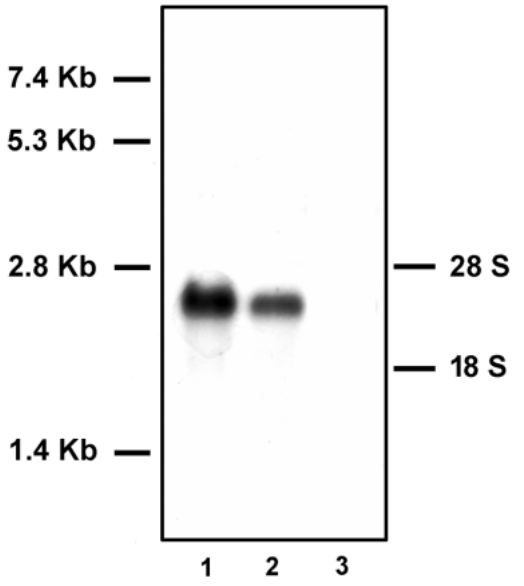

Fig. 2. Northern blot hybridization of ovary and mature oocyte RNA with ^{32}P-labeled probe A (see Materials and Methods). The gel was loaded with 10 μg poly(A)$^+$ (lane 1) and 25 μg of total RNA (lane 2) from ovary and 10 μg poly(A)$^+$ RNA from mature oocytes (lane 3). '28S' and '18S' indicate the migration position of rRNAs. The same hybridization pattern (not shown) was obtained with probe B (see Materials and Methods), corresponding exclusively to the 3′ UTR of the gene, strongly suggesting that the signal observed is not due to hybridization with *hsp70* genes constitutively expressed in somatic tissues (e.g., *hsc70*, *bip*).

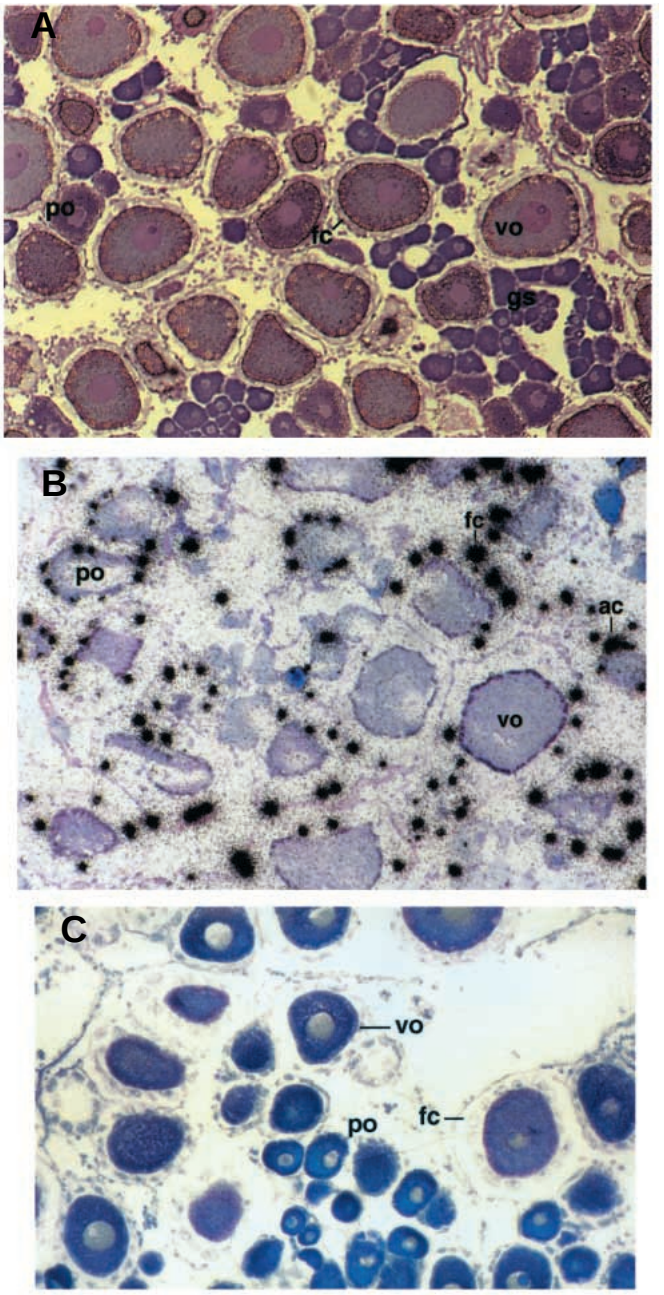

Fig. 3. Expression of the *Cihsp70* gene in *C. intestinalis* ovary. (A) Section of ovary showing the morphology of different stages of oocyte maturation. Sections stained with toluidine blue were viewed with bright-field optics. (B) In situ hybridization of sections of ovary using probe A (see Materials and Methods) indicates that the gene is expressed only in the accompanying cells of young previtellogenic oocytes and in the follicle cells of oocytes that have not undergone GVBD. The use of probe C resulted in the same hybridization pattern. (C) In situ hybridization of sections of ovary using sense probe A. ac, accompanying cells; fc, follicle cells; gs, germinal strand; po, previtellogenic oocyte; vo, vitellogenic oocyte.

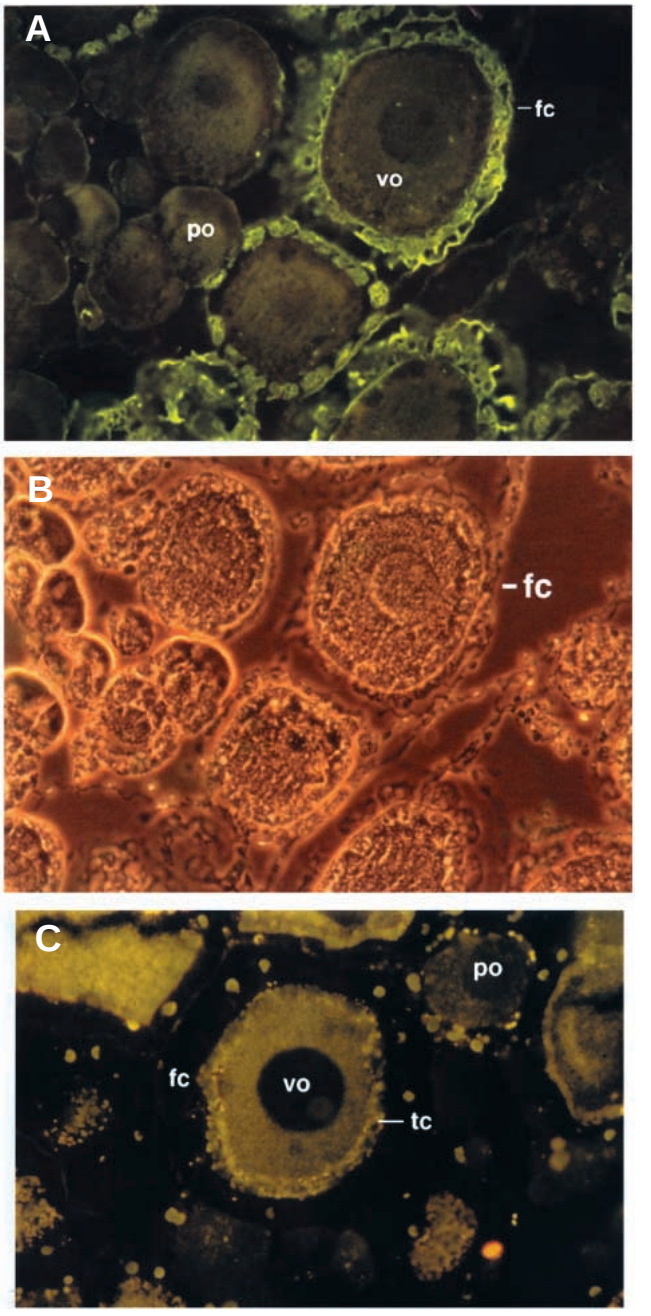

Fig. 4. (A) Localization of Cihsp70 in the ovary as observed by immunofluorescence microscopy. Sections of wax-embedded ovaries were incubated for 2 hours with anti-Cihsp70 polyclonal antibody V15 (Primm, Milano) generated against two peptide sequences located at the COOH-termini of the protein (see Fig. 1). The antibody was used at a dilution of 1:100. Sections were then exposed to biotinylated anti-rabbit IgG and bound antibody was visualized with FITC-conjugated avidin (Sigma). Specific fluorescence is limited to the follicle cells of vitellogenic oocytes. (B) Phase contrast image of the same field shown in A. (C) Immunoflurescence microscopy of an ovary section incubated with the preimmune serum. fc, follicle cells; tc, test cells; vo, vitellogenic oocyte; po, previtellogenic oocyte.

oocytes isolated from the oviduct (Fig. 2); consequently, we suggest that *Cihsp70* plays a functional role during oogenesis.

We conducted in situ hybridization experiments on ovary sections to determine at which stage of oogenesis *Cihsp70* is expressed and by which cell type(s). The previtellogenic stages of oocyte differentiation take place within a germinal strand. When the growing oocyte leaves the germinal strand it is

Table 1. Inhibition of the onset of self-sterility by anti-hsp70 antibodies

Exp. no	Sample	% fertilization* T0†	T3†
1	Con‡	70	0
	V15§	70	70
	Comm¶	70	65
	Pi**	70	0
2	Con‡	50	0
	V15§	50	50
	Comm¶	70	50
	Pi**	50	0
3	Con‡	60	0
	V15d§	60	40
	Comm¶	70	40
	Pi**	60	0

*Percentage of fertilized ovarian oocytes.
†Ovarian oocytes inseminated immediately after dissection (T0) and after an incubation of 3 hours (T3).
‡Control ovarian oocytes.
§Ovarian oocytes incubated with the antibody V15.
¶Ovarian oocytes incubated with the commercial monoclonal anti-hsp70 antibody.
**Ovarian oocytes incubated with preimmune serum.

Table 2. Effect of the anti-hsp70 polyclonal antibody on follicle cells and follicle cell-free oocytes

Exp. no	Sample	% fertilization* T0†	T3†
1	Con‡	100	0
	FC+V15§	100	100
	Ooc.+V15¶	100	0
2	Con‡	50	0
	FC+V15§	50	50
	Ooc.+V15¶	50	0

*Percentage of fertilized follicle cell-free ovarian oocytes.
†Follicle cell-free ovarian oocytes inseminated immediately after dissection (T0) and after an incubation of 3 hours (T3). In both cases follicle cell-free oocytes were incubated with detached autologous follicle cells, separated from the oocytes by dialysis membrane (10,000 molecular weight cut off).
‡Control ovarian oocytes.
§The antibody was added to the follicle cells.
¶The antibody was added to the follicle cell-free oocytes.

accompanied by cells of a non-germinal type that remain flattened on the oocyte surface. At the beginning of vitellogenesis the accompanying cells differentiate into test cells that deepen into the oocyte cytoplasm, and follicle cells that remain at the surface of the oocyte and thus external to the emerging VC (Cotelli et al., 1981). Upon completion of vitellogenesis, GVBD occurs and the test cells are released into the perivitelline space (Fig. 3A). Fully mature oocytes are released into the lumen of the ovary that leads to the oviduct.

Hybridization signals appeared in the accompanying cells of young previtellogenic oocytes and in the follicle cells of vitellogenic oocytes (Fig. 3B). Before GVBD, labeling appeared only in the perinuclear area of the follicle cells of these oocytes. No labeling was found in oocytes at later stages of maturation or in mature oocytes ready for release into the oviduct. The same pattern of expression was obtained with both probes A and C. Therefore, the *Cihsp70* gene is constitutively expressed exclusively in one line of the accompanying cells in ovarian tissue.

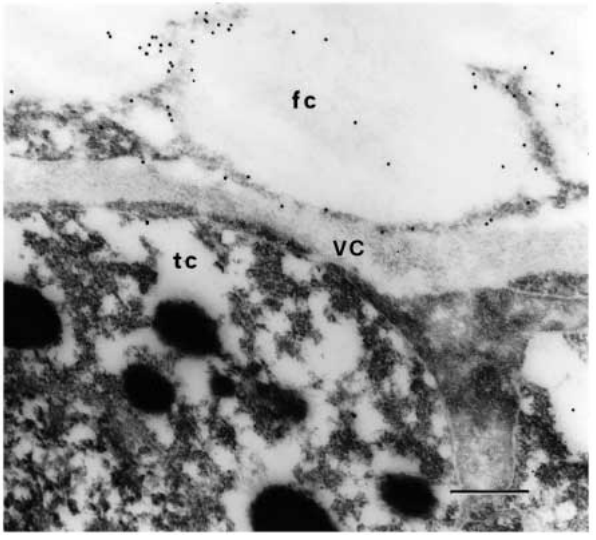

Fig. 5. Immunoelectron microscopic localization of Cihsp70 in a vitellogenic ovarian oocyte. Specific immunolabeling is localized in the follicle cell. Cihsp70 labeling is also evident in the plasma membrane of this cell in the area facing the VC (Scale bar, O.5 μm). fc, follicle cell; tc, test cell; VC, vitelline coat.

Immunohistochemistry

The previous observations are paralleled by immunohistochemical findings obtained in ovarian sections using the polyclonal antibody V15 raised against two 15-amino-acid epitopes located at the most variable region of the C-terminus of the protein (Figs 1, 4). Immunostaining was confined to the follicle cells of vitellogenic oocytes up to GVBD stage. The accompanying cells of previtellogenic oocytes and the follicle cells associated with mature oocytes did not express the

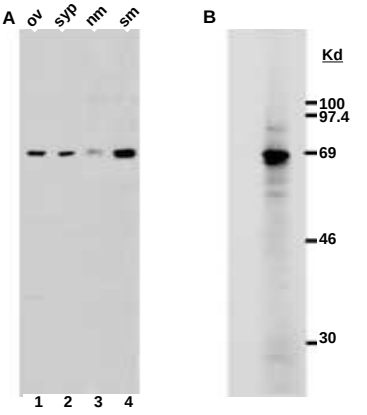

Fig. 6. (A) Immunoblotting of proteins extracted from the gonad (lane 1), muscle (lanes 3 and 4) and of the protein synthesized in vitro (lane 2). The antibody V15 recognizes the protein synthesized in vitro and only one polypeptide band in the ovary extract and in the heat-shocked muscle. (B) SDS-PAGE of the ^{35}S-Cihsp70 protein synthesized in vitro. Molecular size standards are indicated on the right ($M_r \times 10^{-3}$). ov, ovary; syp, in vitro synthesized protein; nm, normal muscle; sm, shocked muscle.

Cihsp70 protein. These results confirm that the expression of Cihsp70 is restricted specifically to one cell line of the ovarian tissue. Furthermore, the timing of expression of the Cihsp70 protein corresponds to the stages of maturation in which the onset of self-discrimination occurs. As observed in other systems (Billoud et al., 1993), the delay between the transcription and translation of the *Cihsp70* gene can be attributed to masking and/or dormancy and storage for future translation.

Transmission electron microscopy of immunogold-labeled sections of ovary revealed the expression of Cihsp70 protein only in the follicle cells of vitellogenic oocytes, restricted to the vacuoles and the cytoplasm bristles that surround them. Interestingly Cihsp70 is also localized at level of the plasma membrane of the follicle cells facing the VC (Fig. 5). No gold particles were found in control sections, incubated either with preimmune serum or with no primary antibody.

Western blot analysis

The study of the expression of the Cihsp70 protein was also carried out by western blot analysis. SDS-PAGE and immunoblotting experiments of the proteins extracted from gonads using the V15 antibody resulted in a single band with a molecular mass of 70,000 (Fig. 6A). The molecular mass of the latter protein estimated by SDS-PAGE coincides with that of the Cihsp70 protein synthesized in vitro (Fig. 6B) and with the theoretical molecular mass calculated from the deduced amino acid sequence. Because the Cihsp70 protein synthesized in vitro was recognized by the V15 antibody (Fig. 6A), the protein recognized in the extracts of gonads was undoubtedly the product of the isolated gene.

To determine whether the gene is inducible, we made an immunoblot analysis of proteins extracted from muscle of heat-shocked and normal animals. The band corresponding to Cihsp70, which was barely detectable in normal muscle, was very prominent after heat shock (Fig. 6A).

These results demonstrate that the gene, inducible in somatic tissues, is expressed under physiological conditions in the gonad during oogenesis.

Biological assay

Oocytes that have undergone GVBD are fertilizable by both autologous and heterologous spermatozoa immediately after isolation from the ovary, and they acquire self-sterility within a maximum of 3 hours when cultured in vitro (De Santis and Pinto, 1991). It has been demonstrated that the ability of the VC to discriminate self spermatozoa involves or is controlled by products of the overlying follicle cells (De Santis and Pinto, 1991; Pinto et al., 1995).

To address the question ‘is hsp70 protein involved in the onset of self-sterility?’ we investigated whether anti-hsp70 antibody interferes with this process. Self-fertile oocytes dissected from *C. intestinalis* ovary were grown to maturity in the presence of the antibody (Table 1). After 3 hours of incubation with the anti-hsp70 antibody (a time sufficient for control oocytes to become self-sterile), oocytes were still receptive to self spermatozoa. The same results were obtained using a commercial monoclonal anti-hsp70 antibody. Neither preimmune serum nor a monoclonal antibody against the follicle cells rendered control oocytes susceptible to fertilization by self spermatozoa. Once established, the self-incompatibility barrier cannot be removed by the antibody as demonstrated when the experiment is carried out on mature self-sterile oocytes (data not shown). Taken together, these data demonstrate that the anti-hsp70 antibody prevents acquisition of self-sterility.

In order to establish whether the effect of the antibody is specifically targeted to the effect exerted by autologous follicle cells, we exploited the finding that follicle cells can induce self-sterility even when they are detached from the VC and left in the medium during oocyte maturation (Pinto et al., 1995). Experiments were carried out on follicle cell-free oocytes that were separated from the homologous detached follicle cells by a dialysis membrane (10,000 molecular weight cut off), a situation that did not prevent the acquisition of self-sterility in the control oocytes. The V15 antibody was added either to the follicle cells or to the follicle cell-free oocytes during maturation. After 3 hours the oocytes incubated in the presence of the V15 antibody became self-sterile like the control. In contrast, the onset of self-sterility was prevented when the follicle cells were incubated with the antibody (Table 2). This confirms that the antibody acts by blocking the function of the follicle cells and consequently by inhibiting the release of self-specific determinants.

DISCUSSION

This study conclusively demonstrates that *Cihsp70* plays a role in the onset of self-sterility in ovarian oocytes of *C. intestinalis*. The *hsp70* gene, a member of the heat-inducible gene family is constitutively expressed exclusively in the oocyte accompanying cells from the early stages of maturation to the completion of vitellogenesis. From early vitellogenesis up to the stages during which self-sterility is established, the hsp70 protein is expressed in the maturing follicle cells. At this stage the use of the V15 antibody prevents the acquisition of self-sterility in the self-fertile oocytes. The fact that the antibody prevents the onset of self-sterility even when the detached autologous follicle cells are separated from the maturing follicle cell-free oocytes by a dialysis membrane, clearly shows that the activity of V15 is exerted directly on the follicle cells and that the products of these cells in turn act on the VC of the maturing oocytes.

Our previous observations showed that the self-incompatibility barrier is established through ‘cross-talk’ between the follicle cells and the VC, which is the final carrier of the determinants for self-nonself discrimination (De Santis et al., 1991). These determinants are the product of a highly specific match between the molecules produced by the follicle cells and those present on the oocyte VC of the same individual (Pinto et al., 1995). This suggests that the factor released by the follicle cells, which is able to modify the VC (i.e. the gateway to non-self spermatozoa), is of a protein nature. In this context hsp70 molecules, as peptide carriers, could be recruited to carry the self-specific components to their counterpart on the VC. It can be hypothesized that hsp70 transports, via a shuttling mechanism, the specificity-endowing molecules from the internal compartments of follicle cells to the cell surface (Fig. 7A). In fact, ultrastructural observations demonstrate the presence of Cihsp70 at the plasma membrane of the follicle cells. This could be accomplished either through association with a fatty acid tail or by binding to a membrane protein. The presence of Cihsp70 at the membrane would elicit the binding

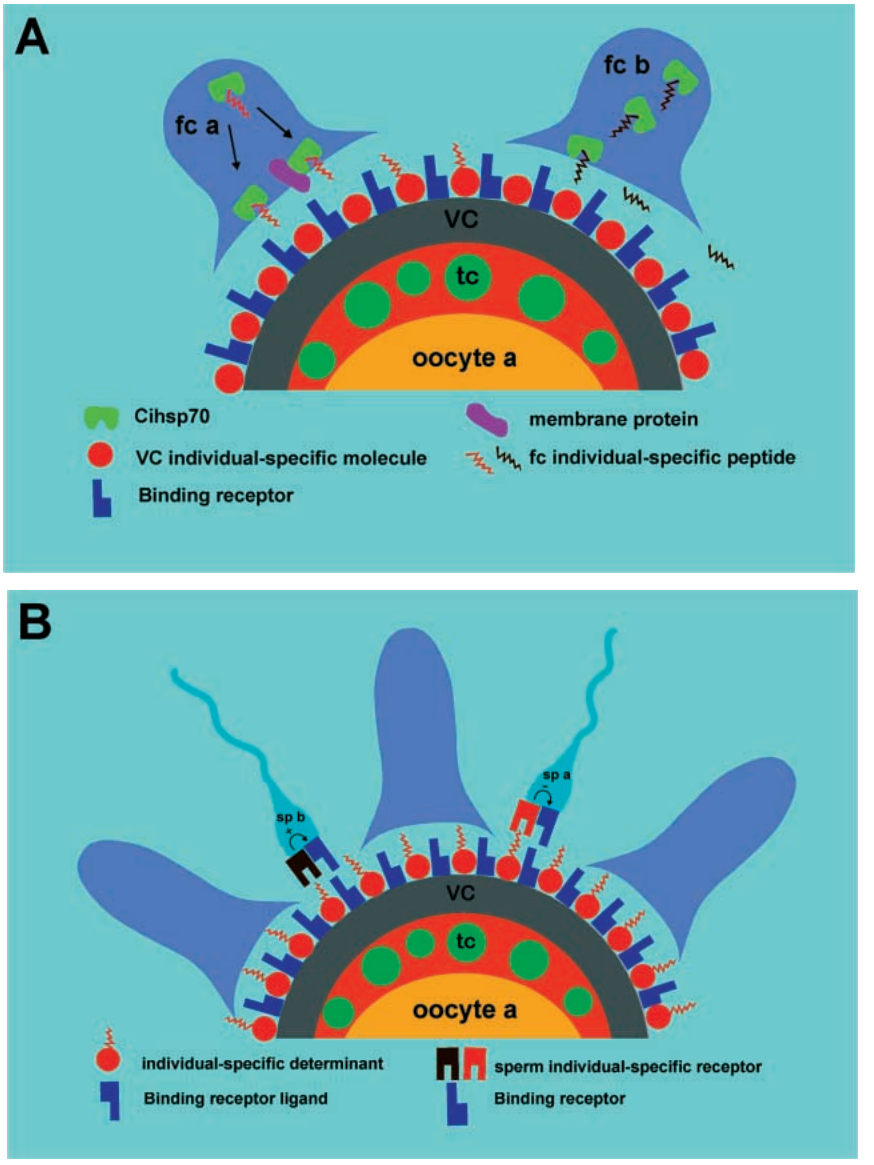

Fig. 7. (A) Schematic representation of the acquisition of self-sterility. Cihsp70 shuttles the individual-specific peptide to an individual-specific molecule at the surface of the VC. This complex represents the histoincompatibility determinant. Cihsp70 is associated with the plasma membrane either through a fatty acid tail (left arrow in follicle cell 'a') or through a plasma membrane protein (right arrow). For clarity in the follicle cell 'b' the anchoring system of Cihsp70 to the plasma membrane is not shown. In the case of the cell b the individual-specific peptide does not bind to the individual-specific molecule at the surface of the VC. (B) Self-nonself discrimination mechanism between sperm and egg. The interaction between the nonself individual-specific determinant on the VC (of oocyte 'a') and the individual-specific receptor on the spermatozoa 'b' enables the binding ligand to interact with its counterpart on the VC. fc: follicle cells; sp: spermatozoon; tc: test cells; VC: vitelline coat.

of the V15 antibody, thus blocking the protein function. Similar pattern and function have been suggested in B-cells and macrophages, i.e. antigen presenting cells (APC), in which a transient cell surface expression of a member of the hsp family has been described. In these cells antibodies specific for a member of the hsp70 family block APC function (Nagel and Pierce, 1991). More recently, it has been observed that human tumor cells, but not normal cells, express an hsp72 molecule on the cell surface and that the cell surface expression of hsp72 correlates with lysability of these tumor cells by NK-like effector cells (Multhoff et al., 1995). Furthermore, hsps isolated from cancer cells or virus-infected cells elicit immune response to the cognate tumor or viral antigens (Suto and Srivastava, 1995).

Self-nonself recognition would occur through the interaction between an individual-specific determinant on the VC and a sperm individual-specific receptor (Fig. 7B). According to our hypothesis, when the VC determinant is engaged by the individual-specific receptor of sperm from the same individual, the sperm binding receptor is inhibited, thus preventing fertilization (Fig. 7B). By contrast, when the sperm individual-specific receptor is not engaged, as occurs in the interaction between spermatozoa with either heterologous or immature oocytes, the binding reaction proceeds.

Even though the mechanism by which hsp70 contributes to the switch from self-fertility to self-sterility remains to be clarified, it must be stressed that fertilization in *Ciona* is an 'all or none' process. Therefore, fertilization is a very sensitive and direct biological assay with which to unequivocally demonstrate the involvement of hsp70 in this process. Lastly, if Cihsp70 indeed exerts, in gamete self-nonself recognition, a function analogous to that of hsp70 in immune recognition, the idea of an ancestral MHC-like system in protochordates begins to take on a firm shape.

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