cDNA Cloning and Functional Analysis of Ascidian Sperm Proacrosin

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In fertilization, sperm must bind to and penetrate through the extracellular glycoprotein matrix surrounding the egg, which is called the zona pellucida in mammals and the vitelline coat in marine invertebrates. After this process, membrane fusion occurs between the sperm and egg. Upon primary binding of the sperm to the vitelline coat, it undergoes an acrosome reaction, which is an exocytosis of the acrosomal vesicle located on the tip of the sperm head. A lytic agent called lysin is exposed on the surface of the sperm head and partially released into the surrounding seawater during the acrosome reaction. In mammals, it has been believed that a trypsin-like enzyme called acrosin (EC 3.4.21.10) is a zona lysin (1, 2). However, recent studies using acrosin-gene knockout mice have revealed that acrosin is not essential for mouse fertilization, although it causes a significant delay (about 30 min) in sperm penetration through the zona pellucida was observed (3, 4). From these results it is currently thought that sperm proteases other than acrosin may participate in sperm penetration through the mammalian egg coat and that acrosin may be involved in the dispersal of the acrosomal matrix (5).

Ascidiens (Urochordata) occupy a phylogenetic position between vertebrates and “true” invertebrates. Although all ascidiens are hermaphrodites, several ascidiens including Halocynthia roretzi are strictly self-fertile. The vitelline-coat lysin system is thought to be activated after the sperm recognizes the vitelline coat of the egg as nonself. To elucidate the roles of sperm proteases in fertilization, we have been studying the sperm proteases of the ascidian H. roretzi, one of the largest ascidiens cultivated for food in Onagawa Bay, Japan. From this animal we can obtain a large amount of sperm and eggs by controlling the seawater temperature and light conditions. Fertilization experiments with this broadcast spawning animal are much easier than those with mammals. We have reported previously that the sperm trypsin-like and chymotrypsin-like proteases are indispensable for sperm penetration through the vitelline coat in H. roretzi by examining the effects of various protease inhibitors on the fertilization of intact as well as naked eggs (6, 7). We have purified two trypsin-like proteases (which we designated as ascidian acrosin and spermoin) from H. roretzi sperm, and we showed that these two proteases play key roles in ascidian fertilization (8–10). The enzymatic properties, including the substrate specificity, of ascidian acrosin are very similar to those of mammalian acrosin. However, to determine whether ascidian acrosin is a mammalian acrosin homologue, information on its amino acid sequence is required.

In the present study, cDNA cloning and functional analysis of ascidian proacrosin were carried out. It was found that ascidian proacrosin has homology to mammalian proacrosin and contains interesting sequences or domains, which were demonstrated to be necessary for proacrosin to bind to the vitelline coat of the egg. To the best of our knowledge, this is the first report showing the existence of sperm acrosin or a mammalian acrosin homologue in invertebrates.

EXPERIMENTAL PROCEDURES

Purification of Ascidian Acrosin—Sperm of the solitary ascidian H. roretzi was collected as described previously (6, 7). Ascidian acrosin was purified according to the procedure described previously (8). The enzymatic activity was determined using 7-butyloxycarbonyl-Val-Pro-Arg-4-methylcoumaryl-7-amide as a substrate (7, 8).

Analysis of the N-terminal Amino Acid Sequence of Acrosin—SDS-
PAGE was carried out in a slab gel (12.5%) according to the method of Laemmli (11). Purified acrosin was subjected to SDS-PAGE and electrochemically transferred to a polyvinylidene difluoride membrane (Millipore). The blotted membrane was stained with 0.1% Coomassie Brilliant Blue R-250 containing 1% acetic acid and 50% methanol. After washing with 50% methanol, the band was cut off from the membrane.

The N-terminal sequence of the purified acrosin was determined using a Procise 492 Protein Sequencer (PerkinElmer Life Sciences).

**Cloning and Sequencing of Ascidian Acrosin cDNA** — The sense primer used for PCR was designed from the N-terminal sequence of ascidian acrosin (5'-GG(T/C/A/G)GA(A/G)TT(T/C)CC(T/C/A/G)TG-3'). This primer encodes the amino acid sequence of CQGDSGGP. The antisense primer encodes the amino acid sequence of CQGDSGGP. The primer was designed from the consensus sequence in the vicinity of the serine residue in the catalytic triad of the trypsin family. The putative sequence of the probe pair was designed from the deduced amino acid sequence of H. roretzi 11. The cDNAs obtained were subjected to cDNA size-fractionation column chromatography following ligation into an EcoRI-digested and phosphorylated 11 vector (Stratagene).

**Northern Blot Analysis** — Total RNA was extracted from each tissue according to the protocol of the manufacturer of the SuperScript Choice System for cDNA synthesis (Life Technologies, Inc.). The cDNAs thus obtained were subjected to cDNA size-fractionation column chromatography following ligation into an EcoRI-digested and phosphorylated 11 vector (Stratagene). Then, in vitro packaging of the cDNA was carried out using a Gigapack III Gold kit (Stratagene).

**Cloning and Sequencing of Ascidian Acrosin cDNA** — The sense primer used for PCR was designed from the N-terminal sequence of ascidian acrosin (5'-GG(T/C/A/G)GA(A/G)TT(T/C)CC(T/C/A/G)TG-3'). This primer encodes the amino acid sequence of CQGDSGGP. The antisense primer encodes the amino acid sequence of CQGDSGGP. The primer was designed from the consensus sequence in the vicinity of the serine residue in the catalytic triad of the trypsin family. The putative sequence of the probe pair was designed from the deduced amino acid sequence of H. roretzi 11. The cDNAs obtained were subjected to cDNA size-fractionation column chromatography following ligation into an EcoRI-digested and phosphorylated 11 vector (Stratagene). Then, in vitro packaging of the cDNA was carried out using a Gigapack III Gold kit (Stratagene).

**Nucleotide and deduced amino acid sequences of H. roretzi proacrosin** — The presumed cleavage sites in preproacrosin are indicated by an arrow and arrowhead, respectively. The deduced amino acid sequence matching the N-terminal sequence of H. roretzi proacrosin is underlined by a solid line. Pairwise basic amino acid residues are indicated by an open square box. The conserved active site residues (His, Asp, and Ser) in the trypsin family are indicated by shaded square boxes. The putative N-linked glycosylation sites are indicated by asterisks. The CUB domains 1 and 2 are underlined by a broken line and a dotted line, respectively.

**In Situ Hybridization** — Ascidian acrosin cDNA was amplified with two primers. The sense primer was 5'-GG(T/C/A/G)GA(A/G)TT(T/C)CC(T/C/A/G)TG-3'.
GGGC-3’, which corresponds to GEMAKLG in the N-terminal sequence, and the antisense primer was 5’-TAAATCAGCTGGCGTTG-3’, which corresponds to SADAADL in the C-terminal sequence. A DNA band migrating at 1010 base pairs was isolated, cloned into a pCRRII vector (Invitrogen), and transformed into E. coli DH5α. An acrosin clone in the pCRRII vector was reacted with EcoRI. An ~1020-base pair fragment was gel-purified from an agarose gel and ligated into a pBluescript SK(+) plasmid vector. The recombinant plasmid was transformed into E. coli DH5α. The sense and antisense cRNAs were synthesized by using T3 and T7 RNA polymerases, respectively. During the synthesis of the cRNAs, digoxigenin-labeled uridine triphosphate was incorporated according to the protocol of Roche Molecular Biochemicals. A gonad of *H. roretzi* was fixed with 4% paraformaldehyde in artificial seawater and embedded in paraffin. Sections of 5 μm in width were prepared. A subsequent enzyme-catalyzed color reaction was conducted by the addition of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt, and the mixture was incubated overnight at room temperature in the dark.

**Preparation of Antibody**—A 14-residue oligopeptide, A2 (RVADLD-KTDDTDEG), corresponding to residues 96–109 in the ascidian acrosin sequence was synthesized and purified by reverse phase high pressure liquid chromatography: A1, AAFLYKHVQVCG (residues 51–62) and A1 (KH/AA), AAFLYAAQVCG. One hundred μl of 1 mg oligopeptide was added to each well of a 96-well plate, and the plate was allowed to stand overnight to adsorb the peptide. After adsorption, each well was blocked with 5% bovine serum albumin and then treated with the biotinylated solubilized vitelline coat. After incubation for 2 h at 4 °C, the plate was washed four times with artificial seawater and incubated with avidin-biotinylated peroxidase complex (Vectastain) in artificial seawater for 30 min according to the manufacturer’s protocol. The plate was washed four times with artificial seawater followed by treatment with 0.05% 3,3′,5′-diaminobenzidine and 0.02% H2O2 to develop the color. Finally, the developed color was measured at 490 nm using a Bio-Rad Model 450 microplate reader.

**Effect of the CUB Domain on Fertilization**—Two 15-residue oligopeptides at residues 348–362 in the CUB domain 1 and 443–456 in the CUB domain 2, respectively, of ascidian proacrosin were synthesized: CUB1 (TEFGVEYHTFCWYDD) and CUB2 (CGEFSSKHYPNYYDA). The eggs were incubated previously with each oligopeptide, CUB1 or CUB2, in seawater for 30 min at 13 °C and were then inseminated. The fertilization ratio was determined as described previously (6, 7).

**Isolation of CUB Domain-binding Components of the Vitelline Coat**—CUB1 or CUB2 peptide-immobilized agarose was prepared using Affi-Gel 10 (Bio-Rad) according to the manufacturer’s protocol. The biotinylated solubilized vitelline coat was incubated with CUB1 or CUB2 peptide-immobilized agarose for 1 h at 4 °C. After washing with artificial seawater, the components of the vitelline coat were eluted with SDS-PAGE sample buffer and subjected to SDS-PAGE. The protein bands were transferred to the polyvinylidene difluoride membrane and subjected to determination of the N-terminal amino acid sequences as described previously.

**RESULTS**

The N-terminal 44-amino acid sequence of the purified acrosin was determined using a protein sequencer (Fig. 1). The N-terminal sequence of acrosin was used to design the degenerate oligonucleotide sense primers for PCR of the *H. roretzi* gonad cDNA library. The antisense primer was designed from the consensus sequence around the active site serine residue of the trypsin family. The deduced amino acid sequence of the PCR product contained the N-terminal peptide sequence of acrosin determined by N-terminal amino acid sequencing. The PCR product was used as a probe for screening the ascidian gonad Agt11 cDNA library to isolate a cDNA clone encoding ascidian acrosin (Fig. 1). The cDNA clone consisted of 2367 nucleotides. A single open reading frame encoded 505 amino acids.
acids. The deduced protein sequence contained a sequence from residues 36 to 79, which corresponds to the N-terminal amino acid sequence determined from the isolated ascidian protein. The molecular mass of proacrosin was estimated to be 55,003 Da. The sequence of the N-terminal 19 residues of proacrosin contained a highly hydrophobic region, probably corresponding to a signal peptide for a nascent protein destined for initial transfer to the endoplasmic reticulum. Thus, the N terminus of proacrosin may start at Asp-20. His, Asp, and Ser, which form a catalytic triad in serine proteases, are located at residues 76, 132, and 227, respectively, in ascidian proacrosin. As compared with mammalian proacrosin, ascidian proacrosin has three consensus sequences for the N-linked carbohydrate chain modification in the C-terminal region but not in the region commonly observed in mammalian proacrosin (e.g., mouse acrosin, Cys-Asn\(^{211}\)-Ser-Thr; ascidian acrosin, Cys-Leu\(^{200}\)-Ala-Thr). Ascidian acrosin also has paired basic residues in the N-terminal region, which are thought to be critical for the binding of acrosin to the zona pellucida (14). In addition, ascidian proacrosin, but not mammalian proacrosin, has two CUB domains in the C-terminal region. It is known that CUB domains are involved in sperm binding to the zona pellucida in the mammalian spermadhesin molecule (15–18).

Northern blot analysis was carried out using the same probe as that used for the screening of cDNA. As shown in Fig. 2, a single transcript of \(\sim 4.7\) kilobases was detected only in the gonads of \(H.\ roretzi\). The expression of proacrosin mRNA was also investigated by \textit{in situ} hybridization. A specific signal was observed in spermatids/spermatocytes (spermatids and spermatocytes are indistinguishable in ascidians by light microscopy) but not in mature sperm with the antisense riboprobe. A specific signal was not observed with the sense riboprobe. These results indicate that ascidian proacrosin mRNA is definitely expressed in both or either of spermatids and spermatocytes (data not shown).

Western blot analysis of the sperm extract demonstrated the presence of 35-, 40-, and 50-kDa proteins that immuno-reacted with the anti-acrosin antibody under nonreducing conditions (Fig. 3). Because proacrosin has a molecular mass of 53,052 Da, the 50-kDa protein seems to be proacrosin. It is inferred that proacrosin with a molecular mass of 53 kDa is processed into the 35-kDa acrosin through the 40-kDa protein as an intermediate.

Acsidian sperm undergoes sperm reaction, accompanied by vigorous sperm movement and mitochondrial shedding, upon sperm binding to the vitelline coat during fertilization (19). This sperm reaction is mimicked by increasing the extracellular pH to 9.0. When sperm of \(H.\ roretzi\) were treated with alkaline artificial seawater (pH 9.0), a similar morphological change was induced. As shown in Fig. 4, ascidian acrosin was found to be released from sperm treated with the artificial seawater (pH 9.0). This result suggests that ascidian acrosin is released from sperm during fertilization. In contrast, proacrosin was not detected in the sperm exudate, suggesting that only the active form of acrosin is released from the reacted sperm.

Ascidian proacrosin contains two interesting sequences: one is the paired basic residues-containing sequence, and the other is the CUB domain. First, the role of the former sequence was investigated. Peptide A1 containing the paired basic residues of ascidian acrosin and the peptide A1(KH/AA), the paired basic residues of which had been substituted with Ala-Ala, were synthesized and tested for their abilities to bind to the isolated vitelline coats. The binding ability of peptide A1 containing the paired basic residues (Lys-His) to the vitelline coat was found to be 2.5-fold higher than that of peptide A1(KH/AA).

Next, the role of the CUB domain was investigated. Ascidian proacrosin contains two CUB domains in the C-terminal region, one being complete and the other being incomplete (Fig. 1). Two peptides called CUB1 and CUB2, which are derived from CUB domains 1 and 2, respectively, were synthesized, and their ability to inhibit fertilization of \(H.\ roretzi\) was tested. As shown in Fig. 5A, the CUB1 peptide inhibited the fertilization more strongly than did the CUB2 peptide. The fact that fertilization is strongly inhibited by the CUB1...
peptide suggests that the CUB domain 1 can interact with the vitelline coat.

To identify the CUB domain 1-binding components of the vitelline coat, a pull-down assay using CUB1 peptide-immobilized agarose beads was carried out. Five components (90-, 85-, 30-, 28-, and 25-kDa proteins) of the vitelline coat were found to be capable of binding to CUB1 peptide-immobilized agarose beads but not to CUB2 peptide-immobilized agarose beads (Fig. 5B). The N-terminal sequences of the 90-, 85-, 30-, and 25-kDa proteins were determined (Fig. 5C). However, we could not find homologous or similar proteins to the above proteins, which are involved in cell-cell interaction as determined by FASTA and BLAST search analyses.

**DISCUSSION**

Here we cloned a cDNA of mammalian acrosin homologue from the solitary ascidian *H. roretzi* (Urochordata), and also we studied a functional analysis of the ascidian acrosin. This is a first report showing the occurrence of acrosin homologue in nonmammalian species. Ascidian acrosin seems to be synthesized as a preproprotein with 505 amino acid residues and with a signal sequence consisting of 19 putative residues in the N-terminal region. By motif search analysis, it was found that ascidian proacrosin has three consensus sequences of N-linked sugar attachment, paired basic residues in the N-terminal region that are thought to be critical for the binding of acrosin to the zona pellucida (14), a protease domain in the middle region, and two CUB domains in the C-terminal region. The amino acid sequences around the active site residues in ascidian acrosin, His76, Asp132, and Ser227, showed high homology to those of the trypsin family including mammalian acrosin (Fig. 6). Identity in the total amino acid sequence between mammalian proacrosins and ascidian proacrosin is 33–35%.
extension (two CUB domains in the case of ascidian proacrosin) of zymogen is a characteristic feature observed in mammalian proacrosin among members of the trypsin family. The purified acrosin gave bands of 34 and 35 kDa under reducing and nonreducing conditions, respectively (data not shown). By analogy to the mammalian acrosin, it is thought that the processed ascidian acrosin probably consists of a short (16 residues) light chain and a heavy chain, both of which are disulfide linked. Taking into account the conserved positions of the cysteine residues in mammalian acrosins, it can be inferred that Cys21 in the light chain is disulfide-bonded to Cys152 in the heavy chain in ascidian acrosin.

The results of the amino acid composition analysis (data not shown) of the purified ascidian acrosin suggested that the C-terminal amino acid is situated in the vicinity of residue 290. If ascidian proacrosin is cleaved autocatalytically, as are mammalian proacrosins, the C-terminal amino acid residue of activated acrosin would be Arg286, because the Arg residues are located at 286, 269, and 254 in the presumed C-terminal region of active acrosin. If it is the case, the C-terminal CUB domains would be completely removed by autocatalytic activation.

In mammalian proacrosin, the participation of the N-terminal region in the binding to the zona pellucida was confirmed by using synthetic peptides and recombinant proteins, and recent studies on site-directed mutagenesis have revealed that the paired basic residues in the N-terminal region of acrosin are crucial for maintenance of the binding of proacrosin to the zona pellucida (14). It was found that ascidian acrosin also has paired basic residues, Lys56-His57, in the N-terminal region that are involved in the binding to the vitelline coat; the binding ability of the peptide containing paired basic residues (Lys-His) to the vitelline coat was found to be higher than that of the peptide containing Ala-Ala. The present findings agree with the above-mentioned finding on the role of paired basic residues in mammalian proacrosin (14).

Several mammalian proacrosins, including human and porcine proacrosins but neither mouse nor rat proacrosin, contain a Pro-rich region in the C terminus that is cleaved off during proacrosin activation (20). Because proacrosin and α-acrosin, unlike the fully activated form β-acrosin, are able to strongly bind to the zona pellucida (21), it is thought that the C-terminal region may also be involved in the binding to the zona pellucida (21). Ascidian proacrosin, but not mammalian proacrosins, has two CUB domains in the C-terminal region. The CUB domain is defined as a 100–110 residue spanning extracellular module, the name of which is derived from the following three proteins: complement subcomponents (Clr/Cl1s) (22, 23), an embryonic sea urchin protein (Uegf) (24), and bone morphogenetic protein I (BMP-I) (25, 26). The most characteristic features of the CUB domain are the existence of three or four conserved Cys residues that form two disulfide bonds and the existence of various hydrophobic and aromatic amino acid residues that participate in the formation of the antiparallel β-barrel topography of the molecule. CUB domains in mammalian spermadhesin are known to be involved in sperm binding to the zona pellucida (15–18). Our present results showing that the C-terminal CUB domain 1 of ascidian proacrosin is involved in the binding to the vitelline coat coincide well with the above-mentioned fact regarding the CUB domain in mammalian spermadhesin. Although ascidian proacrosin does not possess a Pro-rich region in the C-terminal region, the CUB domains seem to work in the binding of proacrosin to the vitelline coat as a functional homologue of the Pro-rich region in mammalian proacrosin.

Of two peptides derived from CUB domains 1 and 2, the peptide of CUB domain 1 strongly inhibited fertilization. Five components (90-, 85-, 30-, 28-, and 25-kDa proteins) in the vitelline coat were found to be able to bind to CUB1 peptide-immobilized agarose beads. It is likely that the CUB1 peptide binds to the vitelline coat, resulting in the inhibition of fertilization. Although we failed to identify the homologous proteins to the above-mentioned five components, it is intriguing to note that the 30-kDa component contains an HAV motif, which is a key element in homophilic cadherin interactions (27). This motif may play an important role in the interaction between the 30-kDa component of the vitelline coat and the CUB domain 1 of ascidian proacrosin. In connection with this, it is also interesting that the CUB domain 2 also contains a His121-Ala242-Val423 sequence. Therefore, we cannot rule out the possibility that CUB domain 2 is also involved in the binding to the vitelline coat.

Concerning the reason why mammalian sperm exposes acrosin and proacrosin after the acrosome reaction, an interesting hypothesis called “binding-releasing mechanism” in mammals has been proposed (28). After the acrosome reaction, exposed proacrosin in sperm binds to the zona pellucida. Once this binding is established, autodigestion of proacrosin takes place, leaving the sperm free to bind again. This sequential mechanism would facilitate sperm penetration through the zona pellucida. In H. roretzi, we could not detect a substantial amount of proacrosin species in the sperm exudate by Western blotting, implying that acrosin released from sperm upon sperm activation may bind to the vitelline coat through the N-terminal paired basic residues and digest the vitelline coat. Alternatively, if the above mechanism in mammals works in ascidians, it can be inferred that the residual membrane-associated proacrosin may bind to the vitelline coat via the C-terminal CUB domain followed by autodigestion, which enables sperm to freely bind to the vitelline coat again. Further detailed study is necessary to clarify this point.

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REFERENCES
1. Muller-Esterl, W., and Fritz, H. (1981) Methods Enzymol. 80, 621–632
2. Urich, U. A., Wardrip, N. J., and Hedrick J. L. (1985) J. Exp. Zool. 233, 481–483
3. Baba, T., Azuma, S., Kashiwabara, S., and Toyoda, Y. (1994) J. Biol. Chem. 269, 31845–31849
4. Adham, L. M., Nayernia, K., and Engel, W. (1997) Mol. Reprod. Dev. 46, 370–376
5. Yamagata, K., Murayama, M., Okabe, M., Toshimori, K., Nakaniishi, T., Kashiwabara, S., and Baba, T. (1998) J. Biol. Chem. 273, 10470–10474
6. Hoshi, M., Numakunai, T., and Sawada, H. (1991) Dev. Biol. 146, 117–121
7. Sawada, H., Yokosawa, H., Hoshi, M., and Ishii, S. (1982) Gamete Res. 5, 291–301
8. Sawada, H., Yokosawa, H., and Ishii, S. (1984) J. Biol. Chem. 259, 2900–2904
9. Sibata, H., Yokosawa, H., Someno, T., Saino, T., and Ishii, S. (1986) Dev. Biol. 105, 246–249
10. Sawada, H., Iwasaki, K., Kihara-Negishi, F., Ariga, H., and Yokosawa, H. (1990) Biochem. Biophys. Res. Commun. 172, 499–504
11. Laemmli, U.-K. (1970) Nature 227, 680–685
12. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
13. Liu, F.-T., Zinnecker, M., Hamaoka, T., and Katz, D. H. (1979) Biochemistry 18, 690–697
14. Richardsen, R. T., and O’land, M. G. (1996) J. Biol. Chem. 271, 24069–24074
15. Calvete, J. J., Sanz, L., Dostaolava, Z., and Topfer-Petersen, E. (1995) FEBS Lett. 334, 37–40
16. Clavete, J. J., Mann, K., Schafer, W., Raida, M., Sanz, L., and Topfer-Petersen, E. (1995) FEBS Lett. 365, 179–182
17. Dostaolava, Z., Calvete, J. J., Sanz, L., Hettel, C., Riedel, D., Schoneck, C., Einspanier, R., and Topfer-Petersen, E. (1994) Biol. Chem. Hoppe-Seyler 375, 457–461
18. Topfer-Petersen, E., and Calvete, J. J. (1996) J. Reprod. Fertil. Suppl. 50, 55–61
19. Lambert, C. C., and Epol, D. (1979) Dev. Biol. 69, 296–304
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20. Baba, T., Kashiwabara, S., Watanabe, K., Itoh, H., Michikawa, Y., Kimura, K., Takada, M., Fukamizu, A., and Arai, Y. (1989) J. Biol. Chem. 264, 11920–11927
21. Urch, U. A., and Patel, H. (1991) Development 111, 1165–1172
22. Leytus, S. P., Kurachi, K., Sakariassen, K. S., and Davie, E. W. (1986) Biochemistry 25, 4855–4863
23. Tosi, M., Duponchel, C., Meo, T., and Julier, C. (1987) Biochemistry 26, 8516–8524
24. Delgadillo-Reynoso, M. G., Rollo, D. R., Hursh, D. A., and Raff, R. A. (1989) J. Mol. Evol. 29, 314–327
25. Bork, P., and Beckmann, G. (1993) J. Mol. Biol. 231, 539–545
26. Romero, A., Roman, M. J., Varela, P. F., Kolln, I., Dias, J. M., Carvalho, A., Sanz, L., Topfer-Petersen, E., and Calvete, J. J. (1997) Nat. Struct. Biol. 4, 783–788
27. Blaschuk, O. W., Sullivan, R., David, S., and Pouliot, Y. (1990) Dev. Biol. 139, 227–229
28. O’land, R., Welch, J. E., and Fisher, S. J. (1986) Adv. Exp. Med. Biol. 205, 131–144