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The human sperm protein PH-20 has hyaluronidase activity

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The PH-20 protein present on the membrane of guinea pig sperm was characterized using a monoclonal antibody [(1991) J. Cell Biol. 111, 2939–2949]. We have isolated the cDNA encoding the human PH-20 protein from a testis library. This cDNA was expressed in RK 13 cells using a vaccinia virus expression system. Cells expressing the human PH-20 protein possess hyaluronidase activity. Treatment with PI-PLC releases the hyaluronidase into the the medium with a concomitant large increase in enzymatic activity. These results demonstrate that the human PH-20 protein has hyaluronidase activity.

PH-20 protein; Hyaluronidase; Human sperm

1. INTRODUCTION

Hyaluronidases have been isolated from such diverse sources as mammalian testis, the venom of snakes, bees and scorpions and the salivary gland of leeches [1]. These enzymes hydrolyze hyaluronan (hyaluronic acid), a polysaccharide of high molecular mass that is found in the extracellular matrix. We have recently determined the amino acid sequence of bee venom hyaluronidase using recombinant DNA techniques [2]. This represents the first structure of a hyaluronidase of animal origin. Surprisingly, it was found that the sequence of this insect enzyme is homologous to PH-20, a glycoprotein present on the head of guinea pig sperm [3].

PH-20 was identified by monoclonal antibodies and has been shown to play a role in the binding of sperm to the egg zona pellucida. This protein is located at the posterior head surface of guinea pig sperm and it migrates to the acrosomal membrane after the acrosome reaction [3,4]. The PH-20 protein is originally synthesized as a polypeptide with an apparent molecular mass of 64 kDa and it is bound to the membrane via a GPI-anchor. During sperm maturation the bulk of PH-20 is cleaved into two domains, an amino-terminal fragment of 41–48 kDa and a carboxy-terminal part of 27 kDa, the two being linked by disulfide bridges [3]. Antibodies against PH-20 have been shown to block fertilization both in vitro and in vivo [5].

Bee venom hyaluronidase and the guinea pig PH-20 protein exhibit 36% identity in a region encompassing approximately 300 amino acids. This led us to hypothesize that PH-20 might be a hyaluronan binding protein or a hyaluronidase.

Here we describe the isolation of a cDNA encoding the human PH-20 protein. The cDNA was expressed in rabbit kidney cells using a vaccinia virus expression system. It could be shown that cells expressing the PH-20 protein possess hyaluronidase activity.

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Abbreviations: GPI, glycosyl-phosphatidylinositol; PI-PLC, phosphoinositide-specific phospholipase C.

2. EXPERIMENTAL

2.1. *Materials*

Restriction endonucleases and DNA modifying enzymes were from Boehringer Mannheim, Bethesda Research Lab., New England Biolabds, or Stratagene. All radiochemicals were from NEN Research Products (Boston, USA). Phosphoinositol specific phsopholipase C was obtained from Sigma (St. Louis).

2.2. *RNA isolation, cDNA cloning, and library screening*

RNA was isolated from guinea pig testis [6] and transcribed into double-stranded cDNA by Moloney murine leukemia virus reverse transcriptase (BRL), and *E. coli* DNA polymerase using standard procedures. A cDNA fragment encoding guinea pig PH-20 protein was then prepared with two synthetic oligonucleotides taken from the published sequence [3] and the polymerase chain reaction. The forward primer was 5′-GATAAAAGGGCACCACCACTC-3′ (positions 460– 480) and the reverse primer was 5′-CAGTTGCCATGAGCCTATAGT-3′ (position 1958–1938, opposite strand). After 30 cycles of PCR, a cDNA-fragment containing about 1500 basepairs was obtained. Partial sequencing confirmed that this cDNA encoded the mature guinea pig PH-20 protein [3]. This cDNA was labeled by the random priming method and then used to screen a cDNA library in λgt11 prepared from human testis (Clontech). Hybridization under low-stringency conditions yielded three positive clones. The insert present in clone CM19 was subcloned into the Bluescript plasmid (Stratagene) and sequenced by the enzymatic method [7] with the Sequenase 2.0 sequencing kit (United States Biochemicals).

2.3. *Preparation of recombinant vaccinia virus*

The cDNA encoding the human PH-20 protein was ligated into the *Sma*I-site of the plasmid pgpt-ATA-18 [8]. The resulting plasmid was

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termed pATA-HPH. Human 143B cells (ATCC CRL8303), an osteosarcoma cell line which lacks thymidine kinase (TK), were infected with 0.1 plaque forming units of wild-type vaccinia virus. After 2 h, a calcium phosphate precipitate of the plasmid pATA-HPH (0.5 μg DNA) was added to the cells and these were then incubated for 2 h at 37°C. During this period, recombination between the viral TK gene and the homologous viral TK-sequences of the plasmid takes place. After addition of fresh medium, cells were incubated for 2 days under non- selective conditions. Cells were then lysed by freezing and thawing. Cultures of about 80% confluent human 143B cells were infected with the lysate and incubated under selective conditions, i.e. in the presence of 5-bromodeoxyuridine (100 μg/ml), mycophenolic acid (25 μg/ml), hypoxanthine (15 μg/ml), and xanthine (250 μg/ml), for 2 days to propagate the recombinant viruses. By plaque assay purification, single recombinant viruses were obtained [8,9]. RK13 cells were infected with recombinant virus and incubated for 24 h at 37°C. In control experiments, cells were infectes with wild-type vaccinia virus.

2.4. *Hyaluronidase assays*

Tests for this enzyme were performed as described by Dorfman [10]. Medium was removed from infected cells and these were then washed once with phosphate-buffered saline. To the cells (ca. 4 × 10 cells), 2 ml of a solution of hyaluronan (Sigma, 125 μg/ml in 300 mM Na-phosphate/35 mM NaCl, pH 5.5, containing 0.005% bovine serum albumin) were added and incubated at 37°C. At different times, aliquots of 0.5 ml were removed, boiled for 5 min, and centrifuged. To the supernatant, 5 volumes of sodium acetate buffer (25 mM, pH 3.75) containing 0.1% bovine serum albumin was added. After 15 min at room temperature, the turbidity was recorded at 600 nm [10].

2.5. *Digestion with PI-PLC*

About 4 × 10 cells were incubated with 0.5 ml phosphate-buffered saline (pH 7.4) containing 1.2 units of PI-PLC for 20 min at 37°C. Subsequently, hyaluronan was added and hyaluronidase activity was determined.

3. RESULTS

3.1. *Sequence of the cDNA encoding human PH-20*

To test whether the PH-20 protein also possesses hyaluronidase activity, we have first isolated the cDNA encoding the mature guinea pig PH-20 using two oligonucleotides derived from the published sequence [3] and the polymerase chain reaction. Attempts to express this cDNA in *E. coli* under conditions which yielded the recombinant bee venom enzyme [2] were unsuccessful. We therefore used the guinea pig cDNA as a probe to screen a cDNA library from human testis. The insert of one positive clone, CM19, was sequenced using the enzymatic method [7] and found to contain 1974 nucleotides excluding the poly(A)-part (see Fig. 1). This cDNA starts with 373 nucleotides of untranslated 5′-end. Interestingly, this region contains six ATG initiation codons, each of which is followed by an in-frame stop codon. A homologous sequence was found in the cDNA encoding guinea pig PH-20 [3] which contains five initiation codons. However, in both instances these upstream ATG codons are not in a favorable context, as deduced by Kozak [11], for initiating translation. After the seventh ATG codon of the cDNA isolated from human testis, an open reading frame is present comprising 509 codons, of which the first 35 probably code for a signal peptide (see Fig. 1). As deduced from the cDNA sequence, the human PH-20 protein contains 474 amino acids. Excluding the signal sequence, the human and the guinea pig PH-20 proteins are about 60% identical in amino acid sequence up to residue 435. However, the structure of the two proteins differs markedly in the segment encompassing the carboxy-terminal 40 residues; moreover, the guinea pig PH-20 protein contains 20 additional amino acids at this end [3].

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..GATAGCAGTGTAGGTGGTTAGCGGCACCTCGGATAAGGTCCTCCTAGGTCAAAGGGAT
GCTAATGACTAGCCGGTGCTCTAGGAAGACATTGAGACCAGCCAACTTCTTGCCTTGATA
ACTACTGAAGAGACATTGGGTGGCTGGATTTTGAAAGCAGACTTCTGGTTATAGGTGATG
CAACTTGAAAAACAATCCTGAAACATGAAACAAGAATAATAATATTTAAATGTAACTTAA
TCATTATACCTCTTTATCCATCAAAGTGGAATTCATTCCATTCCCTTTCATCTGTGCTCA
TACTTTGCATCAGATATTGGGTAAACCAAAGTGTGTAGGAAGAAATAAATGTTTTCATAG
TCATTACACTTTACAATGGGAGTGCTAAAATTCAAGCACATCTTTTTCAGAAGCTTTGTT
                Met G  V  L  K  F  K  H  I  F  F  R  S  F  V
AAATCAAGTGGAGTATCCCAGATAGTTTTCACCTTCCTTCTGATTCCATGTTGCTTGACT
 K  S  S  G  V  S  Q  I  V  F  T  F  L  L  I  P  C  C  L  T |
CTGAATTTCAGAGCACCTCCTGTTATTCCAAATGTGGCTTTCCTCTGGGCCTGGAATGCC
 L  N  F  R  A  P  P  V  I  P  N  V  A  F  L  W  A  W  N  A
CCAAGTGAATTTTGTCTTGGAAAATTTGATGAGCCACTAGATATGAGCCTCTTCTCTTTC
 P  S  E  F  C  L  G  K  F  D  E  P  L  D  M  S  L  F  S  F
ATAGGAAGCCCCCGAATAAACGCCACCGGGCAAGGAGTTACAATATTTTATGTTGATAGA
 I  G  S  P  R  I  N  A  T  G  Q  G  V  T  I  F  Y  V  D  R
CTTGGCTACTATCCTTACATAGATTCAATCACAGGAGTAACTGTGAATGGAGGAATCCCC
 L  G  Y  Y  P  Y  I  D  S  I  T  G  V  T  V  N  G  G  I  P
CAGAAGATTTCCTTACAAGACCATCTGGACAAAGCTAAGAAAGACATTACATTTTATATG
 Q  K  I  S  L  Q  D  H  L  D  K  A  K  K  D  I  T  F  Y  M
CCAGTAGACAATTTGGGAATGGCTGTTATTGACTGGGAAGAATGGAGACCCACTTGGGCA
 P  V  D  N  L  G  M  A  V  I  D  W  E  E  W  R  P  T  W  A
AGAAACTGGAAACCTAAAGATGTTTACAAGAATAGGTCTATTGAATTGGTTCAGCAACAA
 R  N  W  K  P  K  D  V  Y  K  N  R  S  I  E  L  V  Q  Q  Q
AATGTACAACTTAGTCTCACAGAGGCCACTGAGAAAGCAAAACAAGAATTTGAAAAGGCA
 N  V  Q  L  S  L  T  E  A  T  E  K  A  K  Q  E  F  E  K  A
GGGAAGGATTTCCTGGTAGAGACTATAAAATTGGGAAAATTACTTCGGCCAAATCACTTG
 G  K  D  F  L  V  E  T  I  K  L  G  K  L  L  R  P  N  H  L
TGGGGTTATTATCTTTTTCCGGATTGTTACAACCATCACTATAAGAAACCCGGTTACAAT
 W  G  Y  Y  L  F  P  D  C  Y  N  H  H  Y  K  K  P  G  Y  N
GGAAGTTGCTTCAATGTAGAAATAAAAAGAAATGATGATCTCAGCTGGTTGTGGAATGAA
 G  S  C  F  N  V  E  I  K  R  N  D  D  L  S  W  L  W  N  E
AGCACTGCTCTTTACCCATCCATTTATTTGAACACTCAGCAGTCTCCTGTAGCTGCTACA
 S  T  A  L  Y  P  S  I  Y  L  N  T  Q  Q  S  P  V  A  A  T
CTCTATGTGCGCAATCGAGTTCGGGAAGCCATCAGAGTTTCCAAAATACCTGATGCAAAA
 L  Y  V  R  N  R  V  R  E  A  I  R  V  S  K  I  P  D  A  K
AGTCCACTTCCGGTTTTTGCATATACCCGCATAGTTTTTACTGATCAAGTTTTGAAATTC
 S  P  L  P  V  F  A  Y  T  R  I  V  F  T  D  Q  V  L  K  F
CTTTCTCAAGATGAACTTGTGTATACATTTGGCGAAACTGTTGCTCTGGGTGCTTCTGGA
 L  S  Q  D  E  L  V  Y  T  F  G  E  T  V  A  L  G  A  S  G
ATTGTAATATGGGGAACCCTCAGTATAATGCGAAGTATGAAATCTTGCTTGCTCCTAGAC
 I  V  I  W  G  T  L  S  I  M  R  S  M  K  S  C  L  L  L  D
AATTACATGGAGACTATACTGAATCCTTACATAATCAACGTCACACTAGCAGCCAAAATG
 N  Y  M  E  T  I  L  N  P  Y  I  I  N  V  T  L  A  A  K  M
TGTAGCCAAGTGCTTTGCCAGGAGCAAGGAGTGTGTATAAGGAAAAACTGGAATTCAAGT
 C  S  Q  V  L  C  Q  E  Q  G  V  C  I  R  K  N  W  N  S  S
GACTATCTTCACCTCAACCCAGATAATTTTGCTATTCAACTTGAGAAAGGTGGAAAGTTC
 D  Y  L  H  L  N  P  D  N  F  A  I  Q  L  E  K  G  G  K  F
ACAGTACGTGGAAAACCGACACTTGAAGACCTGGAGCAATTTTCTGAAAAATTTTATTGC
 T  V  R  G  K  P  T  L  E  D  L  E  Q  F  S  E  K  F  Y  C
AGCTGTTATAGCACCTTGAGTTGTAAGGAGAAAGCTGATGTAAAAGACACTGATGCTGTT
 S  C  Y  S  T  L  S  C  K  E  K  A  D  V  K  D  T  D  A  V
GATGTGTGTATTGCTGATGGTGTCTGTATAGATGCTTTTCTAAAACCTCCCATGGAGACA
 D  V  C  I  A  D  G  V  C  I  D  A  F  L  K  P  P  M  E  T
GAAGAACCTCAAATTTTCTACAATGCTTCACCCTCCACACTATCTGCCACAATGTTCATT
 E  E  P  Q  I  F  Y  N  A  S  P  S  T  L  S  A  T  M  F  I
GTTAGTATTTGGTTTCTTATCATTTCTTCTGTAGCGAGTTTGTAATTGCGCAGGTTAGCT
 V  S  I  L  F  L  I  I  S  S  V  A  S  L ///
GAAATGAACAATATGTCCATCTTAAAGTGTGCTTTTTCGACTAATTAAATCTTTG[A]n
```

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the insert present in clone CM19 isolated from a human testis library (Clontech). Initiation and in-frame stop codons present in the 5′-untranslated region and the modified polyadenylation signal AT-TAAA close to the 3′-end are underlined, the predicted cleavage site of signal peptidase (score 6.746, see [21]) is marked (|).

3.2. *Expression of human PH-20 cDNA in RK-13 cells using recombinant vaccinia virus*

The cDNA encoding the precursor of the human PH-20 protein was ligated into *Sma*I site of the plasmid pgpt-ATA-18 [8] to yield plasmid pATA-HPH. This vector contains, between two segments of the vaccinia virus thymidine kinase gene, the gene for the bacterial xanthine-guanine phosphoribosyl-transferase [12] under the control of the early/intermediate I3 viral pro-

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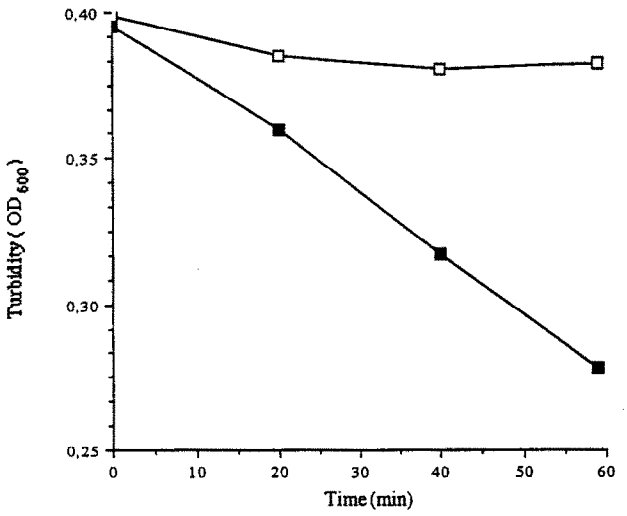

Fig. 2. Hyaluronidase activity of RK13 cells infected with recombinant vaccinia virus (■). As control, cells infected with wild-type virus (-□-) were used. Cells were washed as described in section 2.3 and then incubated with hyaluronan for different periods of time. The decrease of OD-600/ca. 10 cells is plotted against the time of incubation.

moter, and a multiple cloning site downstream of the 11K late promoter.

Recombinant vaccinia virus containing the cDNA encoding the human PH-20 protein was prepared by homologous recombination with the plasmid pATA-HPH. Rabbit kidney RK13 cells were infected with recombinant virus and after 24 h, cells were incubated in the presence of hyaluronan to test for hyaluronidase acitivity [10]. The results of such an experiment are shown in Fig. 2. Hyaluronidase activity was detected on cells infected with recombinant vaccinia virus but not on cells infected with wild-type virus. Essentially the same results were obtained in experiments with crude membranes prepared from cells lysed by freezing and thawing (data not shown).

3.3. *Solubilization of recombinant human PH-20 by PI-PLC*

Proteins bound to membranes via a GPI anchor can be selectively solubilized by incubation with PI-PLC. Upon incubation of RK13 cells expressing the human PH-20 protein with this enzyme, all the hyaluronidase activity was rapidly released from the cells into the medium. Surprisingly, the total amount of activity recovered in the medium was up to ten times higher than that originally detected on the cell surface (see Fig. 3). Again, identical, results were obtained when crude membranes of these infected cells were treated with PI-PLC. In control experiments, no detectable hyaluronidase activity was released under these conditions from cells infected with wild-type vaccinia virus (data not shown).

4. DISCUSSION

Fertilization is a complex process which involves a specific interaction between sperm and egg cells mediated by cell adhesion proteins and the subsequent action of hydrolytic enzymes. PH-20 isolated from guinea pig sperm by using a monoclonal antibody has been shown to mediate binding of sperm to the zona pellucida of eggs. The results presented in this communication demonstrate that human PH-20 also possesses hyaluronidase activity. Similar results have recently been obtained with monkey and mouse PH-20 cDNA expressed in insect cells (P. Primakoff, personal communication). Based on the homology with the bee venom enzyme we assume that the hyaluronidase represents a domain of the PH-20 protein encompassing 350–400 amino acids including 4 conserved cysteine residues [2]. This domain may correspond to the 41–48 kDa fragment of PH-20 present on guinea pig sperm [3]. The PH-20 polypeptide apparently contains a second domain, the 27 kDa carboxy-terminal fragment. There is no counterpart for this part in the soluble bee venom enzyme; moreover, the human and the guinea pig proteins differ both in length and sequence in this region. It is currently not known which parts of the PH-20 glycoprotein are involved in sperm-egg adhesion. One could assume that the variable carboxy-terminal segment is involved in this species-specific interaction.

Like the guinea pig PH-20 protein, its human homologue is also bound to the membrane by a GPI-anchor. Surprisingly, after treatment of RK13 cells expressing the human PH-20 protein with PI-PLC, a 5–10-fold increase in hyaluronidase activity was observed. The reason for this increase in enzymatic activity is presently unknown. A simple assumption would be that the sub-

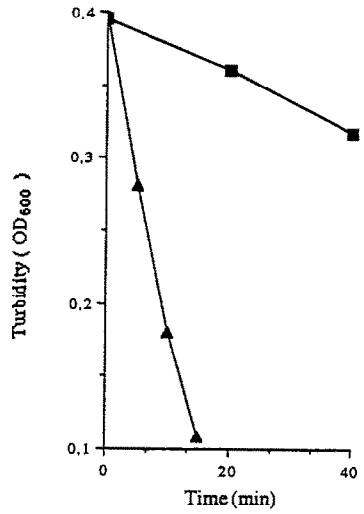

Fig. 3. Release of hyaluronidase by treatment with PI-PLC Cells infected with recombinant vaccinia virus were treated with PI-PLC (see section 2.5). Enzymatic activity, i.e. decrease in OD 600/10 cells, is plotted against time. (-▲-): Hyaluronidase activity after treatment with PI-PLC; (-■-): Control without PI-PLC.

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strate, a polysaccharide with a molecular mass up to several million [13], may have only limited access to the active site of the membrane-bound hyaluronidase due to steric hindrance. In addition, dense clustering of the PH- 20 protein molecules on the cell surface could contribute to this effect. It has been shown that in epithelial cells, GPI-anchored proteins and glycosphingo-lipids tend to form tight complexes that are insoluble in certain detergents [14–16]. A well-studied example is the folate receptor which occurs in dense clusters on the cell surface in association with caveolae [17]. In mature sperm, PH-20 is also clustered in the posterior head region, but it diffuses freely after the acrosome reaction [4,18]. It is currently not known whether this also leads to an increase in hyaluronidase activity of the PH-20 protein.

Many years ago it was shown that mammalian testis is a rich source for soluble hyaluronidases [1]. At present, nothing is known about the structure and biological function of these enzymes. However, hyaluronidases from bovine or ovine testis have, for example, been used for in vitro fertilization to digest the outer layer of cumulus cells which surround the eggs of various mammals. These cells are embedded in a matrix rich in hyaluronan. The present results suggest that upon fertilization in vivo, the hyaluronidase activity of the PH-20 protein enables the sperm to penetrate this outer layer. After this early step, interaction between sperm head proteins, possibly including PH-20, and constituents of the zona pellucida triggers the acrosome reaction [18,19] and fertilization ensues. It has been shown previously that female guinea pigs immunized with the PH-20 protein are sterile [5]. In conjunction with the present results it seems likely that the action of PH-20 as a hyaluronidase is essential for fertilization. This raises the interesting possibility that hyaluronidase inhibitors might also block fertilization and thus function as contraceptives.

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