Title
Characterization of PH-30, a protein with a role in sperm-egg fusion

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Publication Date
1991

Peer reviewed|Thesis/dissertation

CHARACTERIZATION OF PH-30, A PROTEIN WITH A ROLE IN SPERM-EGG FUSION

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Dedicated with love to

my parents,

Gisela,

and Laura and Günter.

ACKNOWLEDGEMENTS

First and foremost I would like to thank Dr. Judith White for being an excellent advisor during the course of my graduate studies. She provided me with a challenging and very interesting project, was always there to discuss new results and to provide inspiration, kept me focused and allowed me a great deal of independance in pursuing our research goals. I really enjoyed working in her lab.

I would also like to thank Drs. Diana Myles and Paul Primakoff for their enormous generosity in providing our lab with the PH-30 project that was the basis for this dissertation. Both have been of invaluable help to us, during my two visits to their lab in Connecticut, and through numerous phone conversations.

I am very grateful to the members of my thesis committee, Drs. Regis Kelly, Zach Hall and Richard Myers, for their help and encouragement over the years. (Reg also threw some great parties).

UCSF has been a stimulating and fun place to be, and the atmosphere of openess contributed strongly to various aspects of my project.

Jeffrey "Sparkles" Glenn put up with me as baymate for five years, during which we enjoyed numerous scientific and political discussions (he pretends to be Republican) and quite a few laughs and outings. Tsafi Danielli, George Kemble and Diane Mason offered scientific advice, but most importantly lots of entertainment. I also enjoyed my collaboration with Tyra Wolfsberg, who introduced me to the wonders of PCR technology. Special thanks also to all other members, present or past, of the White lab.

Chris Turck's help in generating protein sequence information, his patience and encouragement were absolutely essential for cloning both subunits of PH-30.

Eugene "margarita" Napolitano, Pablo "high side" Garcia and Adam "rippin'" Linstedt did their best to show me the ropes in molecular biology. "Rippin'" taught me how to escape (imaginary) sharks by performing the "pearling stop jibe", an advanced windsurfing maneuver. Crissy Fields and the prevailing westerlies remain a special source of inspiration.

I would like to thank Pierre "trailblazer" McCrea and Barry Gumbiner for their input on numerous occasions, and David Julius and members of his lab for primer synthesis and advice in molecular biology.

Peter "prosit" Walter provided a strong incentive to come to UCSF with the promise of good beer and exciting pyrotechnical performances (he kept his promise). I still don't understand why he was made chemical safety officer. Also, beware of playing cards with him as he makes up the rules as he goes along.

Finally I would like to thank "fate" and "luck" for introducing me to Gisela while I was a student here, one of the best things that has ever happened to me.

CHARACTERIZATION OF PH-30, A PROTEIN

WITH A ROLE IN SPERM-EGG FUSION

Carl-Peter Blobel

PhD

Biochemistry

Judith M. White

Chair, Thesis Commitee

Dissertation Abstract

Membrane fusion, both intracellular and extracellular, is vital to all cells. Our working hypothesis is that cellular membrane fusion reactions, like the viral counterparts, are protein mediated. Although the process of viral membrane fusion is mediated by specific well characterized proteins, to date no known bona fide cellular membrane fusion protein has been identified. This dissertation focuses on the guinea pig sperm protein PH-30, which is a good candidate to be a cellular membrane fusion protein mainly because one monoclonal antibody that binds PH-30 inhibits sperm-egg fusion, whereas a second does not.

Biochemical characterization of PH-30 has revealed that PH-30 shares features in common with viral membrane fusion proteins. I showed that PH-30 is an integral membrane glycoprotein composed of two tightly associated and immunologically distinct subunits that are both made as larger precursors. The final processing step of PH-30, which exposes an epitope recognized by the fusion inhibitory monoclonal antibody, coincides with the acquisition of fertilization competence. These results are discussed in terms of the possible role of PH-30 in mediating sperm-egg fusion.

The cloning and sequencing of both mature subunits of PH-30 has lent further support to the proposal that PH-30 is involved in membrane fusion. The N-terminal domain of PH-30 ß was found to be highly homologous to a family of RGD containing snake venom peptides that competitively antagonize platelet aggregation by binding to the platelet integrin IIb/IIIa. We therefore propose that PH-30 ß binds to an integrin on the egg plasma membrane. The α subunit of PH-30 contains a hydrophobic domain with features similar to viral fusion peptides. Both subunits are class I integral membrane glycoproteins. Thus, like many viral fusion proteins, PH-30 is predicted to be a complex of a protein posessing binding activity (ß) and a protein posessing fusion activity (α).

Finally I present evidence for a sperm protease with a possible role in sperm maturation, and for at least two accessory proteins of yet unknown function that copurify with immunoaffinity purified PH-30.

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CHAPTER 1

Introduction and Outline

Membrane fusion is vital to the life of all cells. It is required to unite gametes, such as sperm and egg or yeast cells of opposite mating type, in order to generate a diploid organism. Muscle cells fuse to form the large multinucleated myotubes that support locomotion. Furthermore, enveloped viruses must fuse with their target cells to ensure their propagation. These processes are exoplasmic fusion events, that is between the respective plasma membranes of the fusion partners (reviewed in chapter 2, [1]). All cells also support numerous endoplasmic or intracellular fusion processes. These range from the fusion reactions required to reform organelles after the completion of mitosis to the vesicle fusion processes in the secretory pathway [2].

Despite the abundance of membrane fusion events in nature, very little is known about the mechanism of cellular membrane fusion reactions. Viral membrane fusion, on the other hand, is much better understood. The fusion of virtually all enveloped viruses with their target cells is mediated by viral fusion proteins. These proteins are anchored in the viral envelope through a transmembrane domain. Most viral fusion proteins contain a second hydrophobic domain, in addition to their transmembrane domain, known as the fusion peptide [3]. It is thought that the presence of two hydrophobic domains within a single polypeptide allows the protein to interact simultaneously with the membrane of the host and target cell and thereby to promote the union of the viral and cellular lipid bilayers.

We hypothesize that cellular membrane fusion processes, both exoplasmic and endoplasmic, require fusion proteins conceptually similar to those found in viral fusion reactions. To date, no cellular membrane fusion protein has been described. Our operational criterion for a bona fide membrane fusion protein is the demonstration that the candidate protein promotes membrane fusion in vitro or when introduced into nonfusing cells.

Endoplasmic membrane fusion reactions are being dissected step by step using yeast genetics and biochemical assays with purified subcellular organelles (reviewed in[2]) or semi-intact cells [4]. In both cases, an intricate pathway with many genetically and/or biochemically distinguishable steps has been described. Experiments to date have identified steps leading up to the actual fusion event, which include vesicle budding, vesicle targeting, and the assembly of a "fusion machine". The mechanism of the final fusion event, however, remains to be elucidated.

The main focus of my graduate work has been to search for a cellular exoplasmic fusion protein. Theoretically, this might be accomplished by a variety of means. One could attempt to identify mutants in yeast mating and then screen for those that specifically affect membrane fusion. Using a genetic approach to identify a fusion protein in higher eukariotes would be more problematic due to the lack of simple means to identify defective genes. Therefore, in the latter case a more attractive approach is to raise monoclonal and/or polyclonal antibodies that block fusion. A fusion blocking antibody

would provide an excellent tool to find the cognate protein, which would then be considered a candidate membrane fusion protein. Finally one might attempt to isolate a fusion protein or complex of proteins biochemically. This would require establishing a functional fusion assay in vitro, and fractionating the active components to homogeneity.

We preferred the antibody inhibition approach to search for a cellular fusion protein because of its versatility. It combines a functional assay with a tool to purify candidate proteins for further study. As an experimental system, we initially chose to work with a mouse myoblast cell line called C2. These cells differentiate in culture and fuse to form large multinucleated myotubes. They can be kept in an undifferentiated state by repeated passaging at low cell density and by exposure to high concentrations of fetal calf serum.

In the initial stages of my graduate work, I raised a number of monoclonal antibodies against fusing mouse myoblasts and tested them in an assay designed to monitor myotube formation. This was accomplished by adding myeloma supernatants to muscle cells grown in 96 well tissue culture plates and visually assaying for fusion. None of the monoclonal antibodies I raised appeared to block fusion. Polyclonal rabbit antibodies raised against fusing muscle cells did block fusion. However this was later found to be a consequence of a block in differentiation.

In 1987 a paper was published by Paul Primakoff and coworkers [5] describing a monoclonal antibody that blocked sperm-egg fusion. We wrote to him requesting a sample of this antibody to test on muscle cells. He also sent a control antibody that binds to the same protein but does not inhibit sperm-egg fusion. By our definition this sperm protein, named PH-30 after the fusion inhibitory monoclonal antibody, was a candidate membrane fusion protein. To our great surprise, in initial experiments both the fusion inhibitory and non inhibitory monoclonal antibodies against PH-30 inhibited myoblast fusion. However, this fusion inhibitory effect of the antibodies on muscle cells was not always seen. Unfortunately we were unable to obtain any convincing evidence for the presence of a PH-30 homolog on muscle cells using monoclonal or polyclonal antibodies against PH-30, either in immunoprecipitation studies and Western blots of muscle cell extracts yielded inconclusive results. Instead of further pursuing a muscle PH-30 homolog biochemically, we decided that a more promising approach would be to clone and sequence sperm PH-30 in order to generate a molecular probe for PH-30. This probe could then be used to identify a muscle cell homolog by cross species hybridization analysis. In addition, since PH-30 is a candidate fusion protein in its own right it was also an interesting protein to study in order to learn more about cellular membrane fusion. I therefore switched the focus of my work from muscle cell fusion to sperm-egg fusion and began to work on the structure and function of PH-30.

In chapter 3 [6] I describe a biochemical characterization that revealed features that PH-30 shares in common with viral membrane fusion proteins: It is an integral membrane complex of two glycoproteins α and ß. These proteins are made as larger precursors and are proteolytically processed as sperm matures in the epididymis. Processing correlates with the acquisition of fertilization competence and exposure of epitopes recognized by the fusion inhibitory and noninhibitory monoclonal antibodies.

Chapter 4 describes the cloning of the mature α and ß subunits of PH-30 and discusses some of the implications of the deduced protein sequence. Briefly, we have found that the ß subunit of PH-30 is a class I integral membrane glycoprotein, that is very homologous in its N-terminal region to the snake venom peptide bitistatin, an RGD containing platelet aggregation inhibitor (but does not contain an RGD sequence itself). We believe this result implies that PH-30 is a member of a family of integrin ligands, and is likely to interact with a putative egg integrin. PH-30 might therefore function in both binding of sperm to the egg plasma membrane, and in membrane fusion. Chapter 4 also contains an outline of the cloning of the α subunit of PH-30 (a collaborative project with Tyra Wolfsberg). Like the ß subunit of PH-30, the α subunit is also a class I integral membrane glycoprotein. Interestingly, it shares homology with a viral fusion protein in a hydrophobic domain that we believe could represent a fusion peptide. The results of cloning PH-30 thus lend further strong support to the notion that PH-30 plays an important role in sperm egg fusion. Like several viral fusion proteins (eg. those

of the arbo-, corona-, and bunyavirus families ref), PH-30 is a heterodimer of two class I integral membrane glycoproteins. In these viral fusion proteins, one subunit is involved in binding to the target cell, while the other is involved in fusion. Both the binding and the fusion functions are considered essential to the overall fusion process. Similarly, in the case of PH-30, one subunit (ß) appears to be involved in binding to the target (i. e. egg) membrane, whereas the other subunit (α) appears to be involved in the subsequent fusion reaction.

In chapter 5, a proteolytic activity with a potential role in sperm maturation is described. It was discovered during the study of epididymal sperm maturation (chapter 3). Finally, in chapter 6, two new proteins are described that copurify with PH-30 on a monoclonal antibody column.

The search for a cellular membrane fusion protein has thus led us, starting with muscle cells, to the study of sperm-egg fusion. We hope that with better tools in hand, we will soon be able to return to the study of muscle cell fusion as well, and search for any underlying similarities between the two processes.

CHAPTER 2

Cell-Cell Fusion

(published in Curr. Op. Cell Biol.,1:934-939, 1989)

Introduction

A highly specialized type of cell-cell interaction is plasma membrane fusion. Cell-cell fusion occurs between cells of the same type (homotypic reactions) as well as between cells of different types (heterotypic reactions). Homotypic cell fusion reactions include fusion of myoblasts to form myotubes, fusion of monocytes to form osteoclasts, and fusion of cytotrophoblasts to form the placental syncytiotrophoblast. The products of homotypic cell fusion reactions are multinucleated cells that perform specialized functions. Heterotypic cell fusion reactions occur between gametes. In all cases, cell-cell fusion reactions lead to profound physiological and developmental changes.

Cell-cell fusion reactions are highly regulated. It is imperative that cells fuse only when the environment is correct for their further differentiation and function. It is equally important that cell fusion events be highly specific (i.e., that the fusing partners be of the correct cell type).

Although some of the proteins that regulate and dictate the target specificity of cell fusion reactions have been identified, the actual molecular basis of fusion remains elusive. Since enveloped viruses use specific proteins to mediate their essential membrane fusion reactions, we will first review what is known about viral membrane fusion proteins. We will then propose and discuss a working hypothesis regarding the possible role of proteins in cell-cell fusion

reactions. For more comprehensive recent reviews on this topic see [7] and [3].

The Viral Paradigm

Enveloped viruses infect cells by fusing with cellular membranes. Viral fusion events share an important feature in common with cell-cell fusion reactions. Both processes are "exoplasmic" (Fig. 1); the exoplasmic (outer) leaflets of the fusing bilayers make initial contact. This is in contrast to "endoplasmic" fusion events such as the fusion of transport vesicles carrying material between intracellular organelles. During the latter reactions, the endoplasmic (cytoplasmic) leaflets make initial contact. For a recent review focused on endoplasmic fusion reactions, see [2] Given the differences in the exoplasmic (extracellular) and endoplasmic (cytoplasmic) environments, the mechanisms and proteins involved in exo- and endoplasmic fusion events may differ substantially. Conversely, since cell-cell and virus-cell fusion reactions are exoplasmic, they may share principles in common.

Viral Membrane Fusion Proteins

Two virally-encoded activities are necessary for an enveloped virus to enter its host cell. The first is a specific binding interaction between a viral protein and a host cell receptor; the second is the fusion event itself. For paramyxoviruses (e.g., Sendai), these two functions are contained within separate spike glycoproteins; the HN

glycoprotein mediates binding while the F glycoprotein mediates fusion. For other viruses (e.g. Influenza), the binding and fusion functions reside in the same glycoprotein; however in these bifunctional proteins, the binding and fusion domains are physically distinct (Table I and Fig. 2A,B). We believe that the important segregation of binding and fusion functions observed in enveloped viruses will be reiterated in cellular fusion reactions.

The membrane fusion proteins from members of over 25 different virus genera have now been identified, cloned and sequenced. All that have been analyzed further are oligomeric class I integral membrane proteins in which most of the amino acids (>85%) are external to the virus membrane. Most contain N-linked carbohydrates and many are fatty-acylated. They are present at high density in the viral membranes (e.g., ~3 x $10^4/\mu m^2$ on Influenza). Other than these general similarities, the fusion proteins differ in many important respects (Table I).

Viral fusion proteins can be classified in one of two major categories according to whether they do or do not require exposure to low pH in order to function. Viruses bearing low pH-activated fusion proteins, such as Influenza, are taken into cells by receptor-mediated endocytosis and fuse when they reach an endosome of appropriately low pH (pH 5 to pH 6.5, depending on the particular virus). Conversely, viruses whose fusion proteins function at neutral pH, such as the Human Immunodeficiency virus, are believed to fuse directly with the plasma membrane. Within these two broad groups,

the viral fusion proteins can be further subdivided according to whether or not they possess an identifiable "fusion peptide".

Fusion peptides are currently defined as stretches of apolar amino acids (in addition to the transmembrane domain) that are conserved within but not between virus families. A feature we believe to be functionally important is that all fusion peptides are located in a polypeptide chain which is anchored in the viral membrane (Fig. 2A). Most fusion peptides are found at the amino terminus of the membrane-anchoring chain, whereas several are found internal to the amino terminus. Although prevalent, fusion peptides have not been identified in all viral membrane fusion proteins (Table I). Most of the known fusion proteins are made as larger precursors and then cleaved, late in their biosynthetic pathway, into two polypeptide chains that remain associated through disulfide bonds and/or non-covalent interactions. For most of the cleaved fusion proteins, including those with amino-terminal (e.g., the Influenza hemagglutinin {HA}), internal (e.g., the *env* glycoprotein of Rous sarcoma virus), and no obvious fusion peptide (e.g., the Coronavirus E2 protein), processing appears to be essential for fusion function (Table I).

Our understanding of how a viral protein promotes fusion is most detailed in the case of the Influenza HA, owing largely to the fact that the crystal structure of this molecule is known. The collective findings suggest the following sequence of events: Upon exposure to mildly acidic pH, the trimeric HA spike undergoes a conformational

change. The three globular heads dissociate from one another and the three fusion peptides are liberated from the trimer interface (Fig. 2B). The fusion peptides then interact with lipids of the target membrane. In this manner, the fusion protein is thought to associate simultaneously and hydrophobically with both the viral and target membranes leading to the production of a non-bilayer structure at the contact site (Fig. 2C), a necessary intermediate in the fusion process. Although other viral fusion proteins appear to undergo conformational changes and express hydrophobic moieties under fusion-inducing conditions, we do not yet know whether the fusion mechanism of the HA can be generalized to other viral proteins. We expect that the various viral membrane fusion proteins will exhibit interesting variations on the HA theme, and possibly unexpected mechanisms.

Hypothesis: Specific Proteins Mediate Cell-Cell Fusion Reactions

Fusion is an inherently unfavorable process due to a large energy barrier which prevents membranes from approaching closer than ~20 Å [8]. Because of both this energy barrier and the viral precedent, we propose that, in addition to proteins that regulate and dictate the specificity of cell-cell fusion events, specific proteins facilitate the final bilayer perturbation necessary for cellular union. Variations of this hypothesis have been proposed [7, 9, 10, 11]

Identification of Proteins Involved In Cell-Cell Fusion

For simple viruses such as Influenza and Sendai, the fusion function resides in a single oligomeric spike glycoprotein. For these viruses, assignment of the fusion function has been a relatively straightforward endeavor, involving demonstration of fusion activity following either expression of the cloned gene encoding the fusion protein or reconstitution of the purified fusion protein into artificial vesicles. Identification of the fusion proteins of more complex viruses has proven more difficult. For Herpes simplex virus, which expresses seven different glycoproteins on its envelope, evidence suggests that three separate glycoprotein spikes, gB, gD and gH, may be required for optimal fusion. Given the enhanced complexity of cellular membranes, it is therefore fully anticipated that identification of proteins involved in cell-cell fusion will be a challenging task.

Of the known cell-cell fusion reactions, the two that have been investigated most intensely are myoblast fusion and gamete fusion. Therefore, in the ensuing discussion we will focus on these two cell-cell fusion processes. After discussing proteins that determine the specificity and regulation of these events, we will discuss proteins that have been implicated in reactions more proximal to the final membrane joining.

Proteins Involved In Myoblast Fusion

Knudson and Horwitz proposed that myoblast fusion should be considered as a sequence of events: cell-cell recognition and adhesion and then membrane fusion. As in the viral systems, the binding and fusion steps are biochemically separable events [12, 13].

Specific cell surface molecules are certainly required to provide for the close adhesion and cell type specificity required for myoblast fusion. Two types of cell adhesion systems have been described for both avian [14, 15] and mammalian [16] myoblasts: a calcium-dependent system and a calcium-independent system. Both classes of interactions involve glycoproteins and both may employ multiple components. Likely participants in calcium-independent myoblast adhesion are one or more variants of the N-CAM molecule. This prediction derives from the observation of both qualitative and quantitative changes in the expression of various N-CAM isoforms during myogenesis. Interestingly, the levels of phosphatidylinositol (PI)-linked N-CAM(s) increase during myogenesis *in vitro* [17] and treatment of myoblasts with PI-specific phospholipase C interferes with myoblast adhesion [18]. In terms of calcium-dependent myoblast adhesion, one or more members of the cadherin family [19] are likely to be involved.

Many factors that appear to be inhibitors of myoblast fusion are actually inhibitors of differentiation. For example, a monoclonal antibody against chicken integrin, the extracellular matrix receptor, prevents myoblast fusion by preventing differentiation [20]. This observation, which in its own right is significant and intriguing, highlights an important experimental point. In assessing the effects of inhibitors of myoblast fusion, it is important to determine whether differentiation or a process more directly involved in fusion has been blocked. An additional complication is that myoblast differentiation consists of at least two separable stages, commitment, which is reversible, and terminal differentiation which is not reversible and which, apparently, begins with the onset of fusion [21]. Therefore it would be expected that a fusion inhibitor would block terminal differentiation without blocking commitment.

Several factors have been implicated as regulators of myoblast fusion. Calcium figures prominently in this context. Calcium influx is known to precede membrane fusion [22], and low concentrations of calcium prevent fusion without blocking myoblast differentiation. Calcium channels, such as the embryonic acetylcholine receptor [23], or a recently described stretch-activated Ca^{++}-channel that is most active when myoblasts are fusing [24], are thought to mediate the Ca^{++} influx. The mechanism by which Ca^{++} influx eventually leads to membrane fusion is unclear. Given that prostaglandins [23], phosphatidylinositol bisphosphate [25], protein kinase C and other factors associated with the PI second messenger pathway have also

been implicated (reviewed in [9]), Ca^{++} could well be part of a second messenger pathway that ultimately triggers myoblast fusion.

And what of proteins involved in the final fusion of the myoblast membranes? At present we can only list several candidates. These include a variety of proteins that are developmentally expressed or modified [26, 27, 28]. Although a soluble metalloendoprotease has been implicated in myoblast fusion [11], neither the precise step(s) at which it acts [29] nor its substrates have been identified.

Proteins that maintain lipid asymmetry may be important determinants of the fusogenic capacity of membrane surfaces. The exoplasmic leaflet of chick (but not rat) myoblast plasma membranes contains two to three-fold more phosphatidylethanolamine (PE) and phosphatidylserine (PS) than the exoplasmic leaflets of fibroblast or erythrocyte plasma membranes. Although this enrichment cannot account in total for the fusogenic property of the myoblast surface, it may play an indirect role [30]. In this context it is interesting that an ATPase has recently been described which maintains a relatively high amount of PS in the endoplasmic leaflet of chromaffin granules [31]. It will be interesting to see if chick myoblasts use related mechanisms to maintain relatively high amounts of PS and PE in their fusing surfaces.

Proteins Involved In Gamete Fusion

Gamete fusion is clearly an important cell-cell fusion reaction. It is under study in both lower and higher eukaryotes using a combination of genetic, immunological and biochemical approaches. Clues to some of the proteins involved in gamete binding and fusion are beginning to emerge.

In the baker's yeast *Saccharomyces cerevisiae* two genes, FUS1 and FUS2, have been identified whose products are involved in gamete fusion [32, 33]. Fus1 is an 80kD integral membrane protein which localizes to the gamete attachment site. It has a relatively small extracellular domain with multiple O-linked carbohydrates, a single transmembrane domain, and a relatively large cytoplasmic domain. Characterization of Fus2 is in progress. Future studies are necessary to determine whether either or both of these proteins are involved in breakdown of the cell wall, in binding the plasma membranes of cells of opposite mating types or in the final membrane fusion reaction. A polyclonal antibody blocking approach has been used in preliminary studies of mating in *Dictyostelium discoideum*. Based on adsorption of the fusion-inhibitory activity of a polyclonal antiserum, a protein in the 70 kD range has been implicated in a post-aggregation step required for gamete union [34].

In sperm-egg fusion in higher eukaryotes the initial binding and the fusion reaction are clearly separate events. Initial binding occurs between acrosome-intact sperm and the extracellular glycoprotein coat of the egg, the zona pellucida. Sperm and egg proteins involved in this critical binding interaction have recently been identified and characterized [35]. Following binding to the zona pellucida, sperm undergo the acrosome reaction and migrate toward the egg plasma membrane. Once the sperm and egg plasma membranes meet, the fusion reaction occurs.

The zona pellucida of the egg can be removed such that binding and fusion between (fully differentiated) sperm and egg plasma membranes can be investigated *in vitro*. Various types of perturbants have been used to begin to identify proteins involved in these events. Protease digestion experiments suggest that proteins on the mouse egg plasma membrane may be required for sperm binding [36] Monoclonal antibodies have been used to probe the molecular basis of sperm-egg fusion [37, 38]. Based on these studies, we feel that a good candidate to play a role in sperm-egg fusion is PH-30, a complex of two proteins located in the posterior head region of guinea pig sperm, a region where fusion with the egg occurs. Of two monoclonal antibodies that react with PH-30, one inhibits fusion whereas the other does not. Neither antibody prevents binding between acrosome-reacted sperm and eggs whose zona pellucidae have been removed [5]. Interestingly, inhibitors of metalloendoproteases have been shown to block fusion of sea urchin sperm [37] and human sperm [39] with eggs. As is the case for

myoblasts, the substrates of the implicated metalloendoproteases are not yet known.

Perspectives

Given the energy barrier to fusion we believe that all cellular fusion reactions are, at some level, protein-mediated. More specifically, we speculate that cellular fusion reactions are mediated by membrane fusion proteins. Until proven otherwise we adhere to a rather strict definition of a membrane fusion protein as a protein (or protein assembly) that interacts with lipid components of two apposed bilayers so as to bring about their unification.

To date several proteins have been implicated in cell-cell fusion reactions. However, the precise role of any of these candidates in the overall fusion process remains to be determined. Since cell-cell fusion reactions are exoplasmic, it is tantalizing to speculate that proteins involved in cell-cell fusion events will resemble viral membrane fusion proteins. However, lacking any evidence, at this point in time we must consider alternate possibilities. For example it has been suggested that lysin, a soluble protein from the acrosome granule of abalone sperm, may play a direct role in fusion [40]. It is also conceivable that phospholipid modifying enzymes may play an active role in fusion.

In conclusion, we feel that the most pressing questions in the field of cell-cell fusion are the following: (i) Do specific proteins mediate cell-cell fusion reactions? (ii) If so, do cell-cell fusion proteins share structural features in common with viral membrane fusion proteins? (iii) If specific proteins mediate cell-cell fusion reactions, do they function like viral membrane fusion proteins or (iv) do they employ novel mechanisms to promote the final bilayer destabilization required for membrane fusion? In pursuing these questions it will be important to keep abreast of concurrent progress in understanding viral membrane fusion proteins as well as proteins involved in endoplasmic fusion reactions.

ABBREVIATIONS

HA, hemagglutinin

N-CAM, neural cell adhesion molecule

PE, phosphatidylethanolamine

PI, phosphatidylinositol

PS, phosphatidylserine

Table 1.Fusion proteins of enveloped viruses. For sources of information in this Table and in the text about viral membrane fusion proteins, see citations within reference [3, 7], in press. Yes or no under the heading of "Precursor Processing" refers to whether or not processing of the fusion protein from a larger precursor is required for its fusion function. One of the subunits of the Semliki Forest virus fusion protein is made as a larger subunit; however, in this case, processing does not appear to be required for fusion activity.

Table 1. Fusion proteins of enveloped viruses.

Virus	Binding protein	Fusion protein	Fusion pH	Fusion peptide	Precursor processing
Influenza	HA	HA	Low	N-terminal	Yes
Semliki forest	E123	E123	Low	Internal	No
Vesicular stomatitis	G	G	Low	Not obvious	No
Human immunodeficiency virus	*env*	*env*	Neutral	N-terminal	Yes
Rous sarcoma	*env*	*env*	Neutral	Internal	Yes
Coronavirus	E2	E2	Neutral	Not obvious	Yes
Sendai	HN	F	Neutral	N-terminal	Yes

For sources of information in this table and in the text about viral membrane fusion proteins, see citations within reference [1] and/or in White (*Annu Rev Physiol*, in press). 'Yes' or 'no' under the heading 'Precursor processing' refers to whether or not processing of the fusion protein from a larger precursor is required for its fusion function. One of the subunits of the Semliki Forest virus fusion protein is made as a larger subunit; however, in this case, processing does not appear to be required for fusion activity. F, fusion; HA, hemagglutinin; HN, hemagglutinin/neuraminidase.

Fig. 1 Exoplasmic vs. endoplasmic fusion reactions. Left: depiction of virus-cell fusion as an example of an exoplasmic fusion reaction. Right: depiction of regulated exocytosis as an example of an endoplasmic fusion reaction. The stipled areas represent exoplasmic leaflets and the gray areas represent endoplasmic leaflets of the fusing bilayers. In exoplasmic reactions, the exoplasmic leaflets make initial contact. In endoplasmic reactions, the endoplasmic leaflets make initial contact. Other examples of exoplasmic and endoplasmic fusion events are listed.

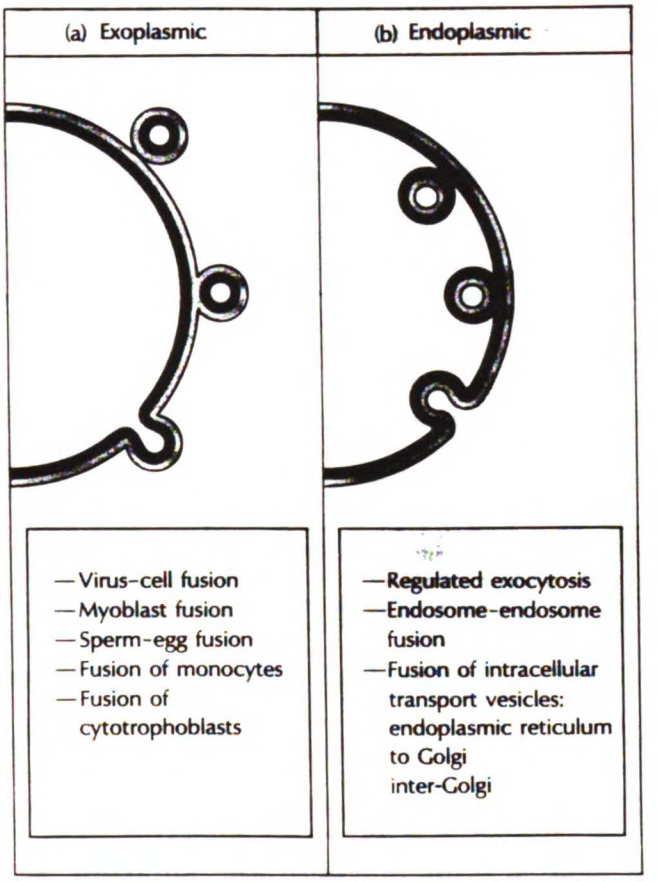
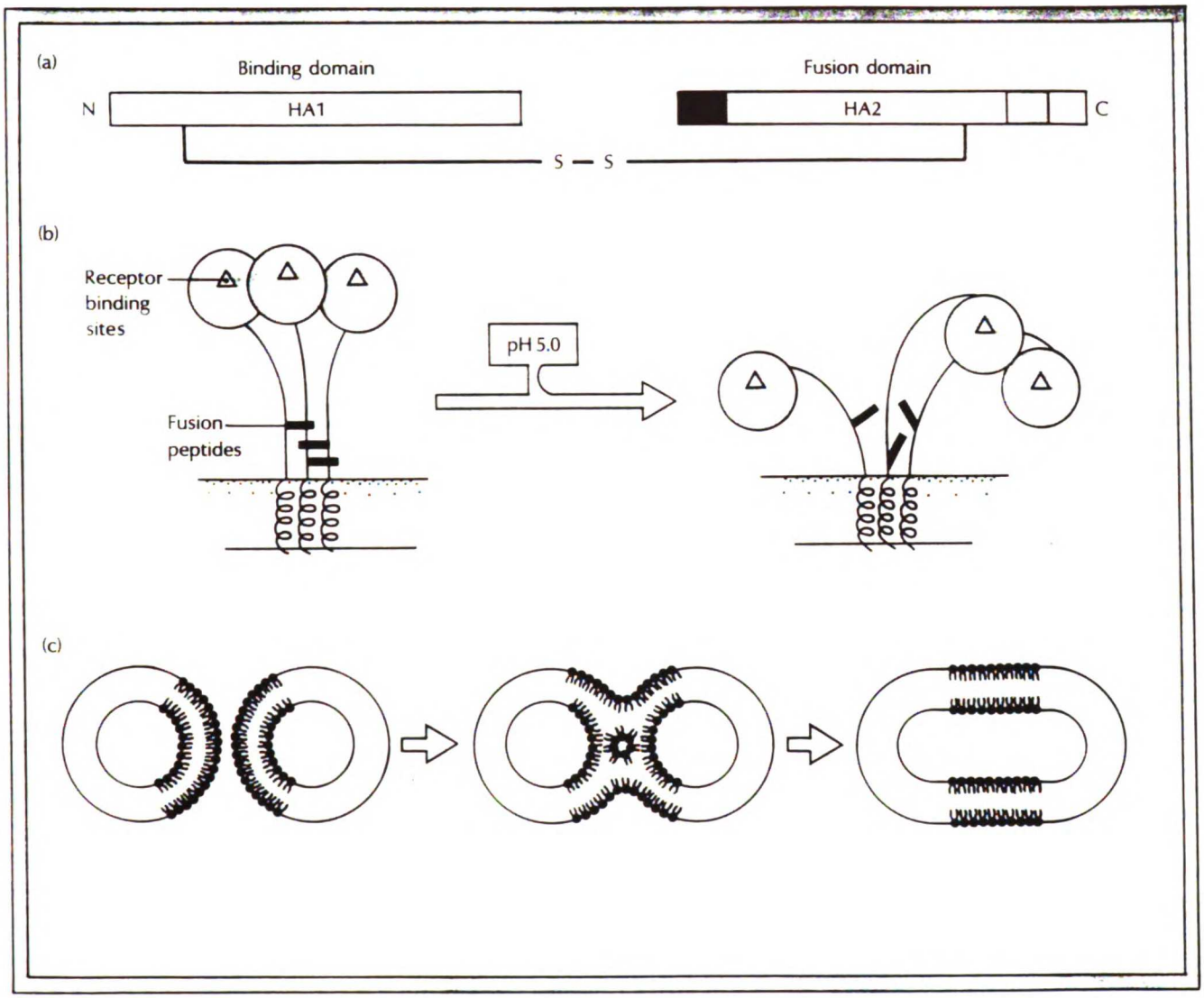

(a) Exoplasmic
(b) Endoplasmic
—Virus–cell fusion
—Myoblast fusion
—Sperm-egg fusion
—Fusion of monocytes
—Fusion of cytotrophoblasts
—Regulated exocytosis
—Endosome-endosome fusion
—Fusion of intracellular transport vesicles: endoplasmic reticulum to Golgi
inter-Golgi

Figure 2. Proposed fusion mechanism of the Influenza HA. (A) The Influenza HA is a trimer composed of three identical subunits, HA1-S--S-HA2. The receptor binding site is located in the HA1 polypeptide while the fusion peptide is located in the HA2 polypeptide, the chain that anchors the protein into the virus membrane. (B) The trimer projects as a spike from the virus membrane. The HA1 polypeptide which houses the receptor binding site forms a globular domain that rests upon a fibrous stalk which houses the fusion peptide. In the neutral pH conformation, the first ten residues of the fusion peptide are buried inside the trimer interface. After exposure to low pH, the globular heads dissociate from one another and the fusion peptides are released from the interface. The released fusion peptides are then capable of interacting with lipid components of the target membrane. For details see reference [3, 7], and citations within. (C) Steps in a membrane fusion reaction. Left: the bilayers approach each other closely. Middle: A non-bilayer structure is formed at the contact site. Right: The unstable non-bilayer intermediate breaks in the plane perpendicular to the original bilayers so as to form one united bilayer. How the partially unfolded HA causes the formation of a non-bilayer structure is under active investigation.


(a)
Binding domain
Fusion domain
N
HA1
HA2
C
S — S
(b)
Receptor binding sites
Fusion peptides
pH 5.0
(c)

CHAPTER 3

Proteolytic Processing of a Protein Involved in Sperm-Egg Fusion Correlates with Acquisition of Fertilization Competence

(published in J. Cell Biol. 111:69-78, 1990)

ABSTRACT

A protein located on the surface of guinea pig sperm (PH-30) has been implicated in the process of sperm-egg fusion [5]. In this paper we have assessed basic biochemical properties of PH-30 and have analyzed the molecular forms of PH-30 present at different stages of sperm maturation. We show the following: (i) PH-30 is an integral membrane glycoprotein. (ii) It is composed of two tightly associated and immunologically distinct subunits. (iii) Both subunits are made as larger precursors. (iv) Processing of the two subunits occurs at different developmental stages. (v) The final processing step occurs in the region of the epididymis where sperm become fertilization competent. (vi) Processing can be mimicked *in vitro*. (vii) Processing exposes at least two new epitopes on PH-30; one of the newly exposed epitopes is recognized by a fusion-inhibitory monoclonal antibody. It has recently been shown [41] that processing also leads to localization of PH-30 to the posterior head, a region of the sperm plasma membrane involved in fusion with the egg. These results are discussed in terms of the possible role of PH-30 in mediating fusion with the egg plasma membrane.

INTRODUCTION

Membrane fusion is ubiquitous in nature. Endoplasmic fusion reactions include fusion of endocytic vesicles, exocytic vesicles and vesicles mediating other aspects of interorganelle traffic. Exoplasmic fusion events include viral fusion, myoblast fusion and fertilization. The fusion events that are best understood at a molecular level are those of enveloped viruses. Viral fusion reactions are mediated by specific virally-encoded membrane fusion proteins. These are integral membrane glycoproteins that exist as oligomers on the virion surface. A large number of viral fusion proteins are made as precursors and then cleaved into two subunits that remain associated through disulfide bonds and/or non-covalent interactions. For fusion proteins consisting of two subunits derived from a common precursor, cleavage is essential for fusion activity. Most, but not all, viral fusion proteins possess a 'fusion peptide', a stretch of apolar amino acids that is thought to initiate fusion by interacting with lipid components of the target bilayer. For recent reviews on membrane fusion and membrane fusion proteins see [1, 2, 3, 7].

Although it has been hypothesized that cellular fusion events are also protein-mediated, the identities of cellular membrane fusion proteins and their modes of action remain elusive. Several proteins involved in steps leading to endoplasmic fusion events have been identified and partially characterized [10, 42, 43, 44]. Less, however, is known about proteins that mediate exoplasmic fusion events occurring between cellular membranes [45].

We have set out to study the molecular mechanism of gamete fusion, an exoplasmic fusion reaction common to all eukaryotic organisms. A protein that has been strongly associated with the process of gamete fusion is PH-30, a protein found in the posterior head of mature guinea pig sperm. Of two monoclonal antibodies that bind to PH-30, one inhibits fusion whereas the second does not. Neither antibody prevents sperm from binding to the egg plasma membrane [5]. Due to its potential role in the overall fusion process we have characterized biochemical features of PH-30. In the course of this work we established that PH-30 is made as a larger precursor and that processing of PH-30 during sperm maturation correlates with the acquisition of fertilization competence.

RESULTS

The α and β subunits of PH-30 are immunologically distinct

A monoclonal antibody, mAb PH-30, blocks fusion of acrosome-reacted guinea pig sperm with zona-free guinea pig eggs. This antibody immunoprecipitates two protein chains of M_r 60 kD and 44 kD on reducing SDS-PAGE [5]. When these polypeptides are boiled, but not reduced, their apparent masses on SDS polyacrylamide gels are ~ 45 kD and ~27 kD, respectively. In this report, the larger of the two polypeptides will be referred to as the α chain and the smaller as the β chain.

Differences in peptide fragments released from α and β following digestion with V8 protease have suggested that α and β are not related [5]. An immunological approach confirmed this finding. A rabbit polyclonal antiserum was raised against PH-30 protein that had been purified, in a nonreduced and nondenatured form, on a column containing mAb PH-30. Antibodies against the individual α and β chains were micro-affinity purified as described in the Methods section. The complete antiserum and the affinity-purified α and β antibodies were then used to probe immunoblots of sperm cell extracts. Since the polyclonal antibody does not react well with reduced PH-30 protein, all immunoblots presented here are of nonreduced cell extracts.

When used to probe an extract of epididymal sperm, the unfractionated polyclonal antiserum recognized both the α chain and the β chain of PH-30 (Fig. 1 A, lane 1). The apparent molecular weights of these subunits were ~45 and ~27 kD, respectively. These values differ slightly from those reported previously [5], since they were calculated with reference to prestained molecular weight markers. Affinity-purified antibodies against the α chain reacted only with the α chain (Fig. 1 A, lane 2); affinity-purified antibodies against the β chain reacted only with the β chain (Fig. 1 A, lane 3). The two chains are therefore immunologically distinct. Both the fusion inhibitory monoclonal antibody, mAb PH-30, as well as the control monoclonal antibody, mAB PH-1, coimmunoprecipitate α and β from nondenaturing detergent cell lysates [5]. Immunoblot analysis with mAb PH-1 and mAb PH-30 revealed that both of these monoclonal antibodies react with the β subunit of PH-30 (Fig. 1 A, lanes 4 and 5, respectively). It is therefore plausible that mAb PH-30 inhibits fusion by binding to a functionally important epitope of the β chain of PH-30.

Multiple β chains exist on mature sperm

Under non-reducing conditions the α chain migrates as a broad band, and the β chain migrates as a doublet. However, if purified PH-30 is analyzed by SDS-PAGE under reducing conditions, the α chain runs as a single sharp band. The β chain, however, retains its appearance as a doublet even under reducing conditions (not shown). On non-reducing SDS-PAGE the β doublet appears as a strong band of

apparent MW 25 kD and a weaker band of apparent MW 28 kD (Fig. 1A, lanes 3-5). The upper β band (28 kD) is apparently composed of two species. To show this, a sperm extract was immunodepleted of mAb PH-30 reactive material as described in the Methods section. The mAb PH-30 immunodepleted extract was then incubated with Sepharose beads coupled with IgG from the rabbit polyclonal antiserum. The bound material was subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with affinity-purified β-specific polyclonal antibodies. This procedure revealed a single band which we will call β_1 (M_r 28 kD, Fig. 1A, lane 6). The β_1 band comigrated with (or ran slightly slower than) the upper of the two bands of material that react with mAb PH-30, which we will term β_2 (M_r 27-28 kD, Fig. 1A, lane 7). The upper band of what appears as a doublet when whole sperm extracts are blotted with the polyclonal antiserum will therefore be referred to as $\beta_{1,2}$ (Fig. 1A, lanes 1 and 3). We term the lower band reactive with mAb PH-30, β_3 (M_r 25 kD, Fig. 1A, lane 7). Thus, on mature epididymal sperm there appear to be at least three β subunits, β_1, β_2, and β_3. All three β subunits are recognized by polyclonal anti-PH-30 antibodies on immunoblots (Fig. 1A, lanes 6 and 7). Preimmune rabbit serum (Fig. 1A, lane 8) and non-specific mouse IgG (Fig. 1A, lane 9) did not bind to any material comigrating with the PH-30 bands. At present the functional significance of the various β species found on mature epididymal sperm is not clear.

PH-30 is a tight complex of the α and β chains

Since both monoclonal antibodies, mAB PH-30 and mAB PH-1, coimmunoprecipitate the α and β chains of PH-30 (14), but bind only to the β chain on immunoblots (Fig 1 A lanes 3 and 4), it appears that α and β form a complex. Further evidence that this is, indeed, the case was obtained by analyzing extracts of epididymal sperm that were treated with SDS at RT (rather than at 100°C) prior to electrophoresis on non-reducing SDS-PAGE. When a sample treated with 0.5% SDS at RT was subjected to SDS-PAGE and then blotted with the polyclonal PH-30 antiserum, the only band detected was of apparent MW 75 kD (Fig. 1B, lane 1). Affinity-purified antibodies against both the α chain and the β chain recognized the 75 kD band (Fig. 1 B, lane 2 and 3, respectively). The 75 kD band was not recognized by the rabbit preimmune antiserum (Fig. 1 B, lane 4). The 75 kD band thus represents a complex of the α and β chains. This complex was completely stable in 0.5% SDS at RT and largely stable (≥75%) in SDS concentrations as high as 4% at RT. PH-30 of epididymal sperm thus appears to be a tight but not covalently-linked complex of two distinct subunits, α and β.

PH-30 is an integral membrane glycoprotein

In order to determine whether the α and β chains of PH-30 are glycoproteins, mature epididymal sperm extracts were incubated with Con-A Sepharose. The bound glycoproteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the polyclonal antiserum against PH-30. Both the α and the β chain bound quantitatively to Con-A Sepharose CL-4B (Fig. 2 A, lane 1). No PH-30 remained in the Con-A Sepharose CL-4B supernatant (Fig. 2 A, lane 2). Control Sepharose CL-4B beads did not bind PH-30 (Fig. 2 A, lane 3), and therefore both chains remained in the Sepharose CL-4B supernatant (Fig. 2 A, lane 4). These data suggest that at least one subunit of PH-30 is a glycoprotein.

To determine which chain(s) of PH-30 contain N-linked carbohydrate moieties, a mature epididymal sperm extract was treated with endoglycosidase F, subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted with the polyclonal PH-30 antiserum. After endoglycosidase F treatment, the mature α and β chains of PH-30, which normally migrate at 45 and 25-28 kD respectively (Fig. 2 B, lane 1), migrated with molecular weights of 38 kD and 21-24 kD, respectively (Fig. 2 B, lane 2). Therefore, both the α and the β chain of PH-30 contain N-linked carbohydrates.

To assess whether PH-30 is an integral membrane protein, its extractability from mature epididymal sperm by high salt (0.5 M KCl) or alkali (0.1 M $NaHCO_3$, pH 11.5) was monitored (6). Sperm were incubated for 1 hr on ice with either high salt or alkali and then pelleted at 200,000 x g for 30 min. Pellets of the extractions with 0.5 M KCl, 0.1 M KCl (as control), and 0.1M $NaHCO_3$ were run on SDS-PAGE (Fig. 2 C, lanes 1, 3, 5 respectively) next to their corresponding supernatants (Fig. 2 C, lanes 2, 4, 6 respectively), transferred to nitrocellulose and immunoblotted with the polyclonal PH-30 antiserum. In each case, PH-30 was quantitatively recovered in the membrane pellet fraction; no PH-30 was detected in the respective supernatants. PH-30 is therefore resistant to extraction by either high salt or alkaline conditions. These results, together with the binding of PH-30 to Con-A Sepharose and the removal of N-linked carbohydrate chains after endoglycosidase F digestion, show that PH-30 is an integral membrane glycoprotein.

The PH-30 α and β chains are made as larger precursors.

We next determined the molecular forms of the α and β subunits on sperm at different developmental stages. Two populations of testicular cells were analyzed: a pool of all testicular spermatogenic cells largely depleted of testicular sperm (referred to here as testicular cells) and isolated testicular sperm, the most fully developed cells in the testis. In addition we analyzed sperm from three stages of epididymal maturation: the distal corpus-, the

proximal cauda-, and the distal cauda epididymis. In the three rodent species analyzed to date (rat, mouse and hamster), fertilization competence is first observed in the distal corpus epididymis and then increases dramatically in the proximal cauda epididymis [46, 47]. Cell extracts were separated by SDS-PAGE and subjected to immunoblot analysis with affinity-purified antibodies against the individual α (Fig. 3, A and C) or β subunits (Fig. 3, B and D). One set of extracts was boiled in 2% SDS to detect the individual subunits (Fig. 3, A and B), whereas another set was treated with 0.5% SDS at RT to resolve complexes of α and β (Fig. 3, C and D, see also Fig. 1 B).

As seen in Fig. 3A, α was present on testicular cells predominantly as a precursor, pre α, of molecular mass 105 kD although some mature α was also visible (Fig. 3A, lane 1). However, on testicular sperm (Fig. 3 A, lane 2), only the mature α chain (45 kD) was present. This implies that the pre α chain (105 kD) is processed to mature α (45 kD) in the testis before the testicular sperm stage. The size of the α chain does not change upon continued passage through the distal corpus epididymis (Fig. 3 A, lane 3) or the proximal (Fig. 3 A, lane 4) or distal cauda epididymis (Fig. 3A, lane 5).

The maturation pattern of β was quite different from that of α. On testicular cells antibodies specific for β detected a precursor, pre β, that appeared as a doublet with an average molecular mass of ~85 kD (Fig. 3B, lane 1). On testicular sperm, β antibodies detected a single band migrating at 88 kD, slightly higher than the doublet on

testicular cells (Fig. 3B, lane 2). Unlike α, however, β did not appear to be proteolytically processed in the testis. Rather, it was observed to be processed separately and later during passage of sperm through the epididymis. In the distal corpus epididymis (Fig. 3 B, lane 3), β antibodies detected a 75 kD band that we will refer to as pre β*, and a band at 28 kD corresponding to β_1 and/or β_2. Mature β_3 (25 kD), in addition to β_1/β_2 (28 kD), was found only in the proximal and distal cauda epididymis (Fig. 3 B, lanes 4 and 5, respectively).

As shown in Fig. 1B, complexes of α and β can be resolved when extracts of distal cauda epididymal sperm are treated with 0.5% SDS at RT and run on nonreducing SDS-PAGE. Analysis of nonboiled and nonreduced samples of testicular cells, testicular sperm, and sperm from the epididymis (distal corpus, proximal cauda, distal cauda) revealed complexes of the respective PH-30 precursors. These precursors were recognized by both α and β specific antibodies (Fig. 3 C and D, respectively).

On unboiled testicular cells, α and β antibodies detected bands of molecular mass 190 and 160 kD, (lane 1 in Fig. 3 C and D, respectively). The 190 and the 160 kD bands therefore most likely represent complexes of the PH-30 α and β precursors. The presence of uncomplexed pre β on testicular cells (Fig. 3 D, lane 1) suggests that pre β might be present in excess of pre α.

On unboiled testicular sperm, the α and β antibodies detected only one band at 160 kD (Fig. 3 C and D, lane 2). We established above that testicular sperm contain only pre β and mature α (Fig. 3 A and B, lane 2). Thus, the 160 kD band represents a complex of α and pre β. We therefore interpret the 190 kD band seen on nonboiled testicular cells to be a complex of pre α and pre β.

On unboiled extracts of sperm from the distal corpus epididymis two bands, at 140 kD and 75 kD, were recognized by affinity-purified α and β antibodies (Fig. 3 C and D, lane 3). Sperm from the proximal and the distal cauda epididymis contained only one band at 75 kD (Fig. 3 C and D, lanes 4 and 5, respectively, see also Fig. 1 B). Given that the 75 kD band represents a complex of mature α and β, the band at 140 kD on distal corpus sperm must be a complex of pre β* with mature α. Collectively these results illustrate that both the α and the β chain of PH-30 are made as larger precursors. Complexes of pre α and pre β exist at early developmental stages. Proteolytic processing of the α and β chains occurs independently at different stages of sperm maturation.

In vitro proteolysis of testicular sperm leads to processing of PH-30

We next wished to determine whether the precursors of PH-30 could be converted to their mature forms by proteolysis *in vitro*. Untreated testicular sperm (Fig. 4A and B, lane 1) were run on SDS-PAGE next to testicular sperm that had been treated on ice with either proteinase K, chymotrypsin, trypsin or the metalloendoprotease, dispase (Fig. 4, A and B, lanes 2-5, respectively). The protease-treated testicular sperm samples were then subjected to immunoblot analysis with β and α specific antibodies (Fig. 4, A and B respectively).

All four proteases tested affected the size of pre β found in testicular sperm extracts. Treatment with proteinase K yielded bands at ~47 kD (Fig. 4 A, lane 2). Treatment with chymotrypsin almost quantitatively converted the pre β chain to a 30 kD band (Fig. 4 A, lane 3) that migrated slightly above $\beta_{1,2}$ on mature distal cauda epididymal sperm (Fig. 4 A, lane 6). Treatment with trypsin (Fig. 4 A, lane 4) generated a 28 kD band comigrating with $\beta_{1,2}$ on epididymal sperm. Finally, treatment with the metalloendoprotease dispase (Fig. 4 A, lane 5) converted pre β to a 27 kD band migrating between $\beta_{1,2}$ (28 kD) and β_3 (25 kD) on mature sperm. Thus, pre β of testicular sperm can be converted into lower molecular mass species by proteolysis. Treatment with chymotrypsin, trypsin or dispase resulted in products that were similar in size to the β chains found on mature epididymal sperm. A 20 fold increase in concentration of

these three proteases did not lead to further processing or degradation of β (not shown). In contrast to their effects on pre β, treatment of testicular sperm with proteinase K, chymotrypsin, trypsin or dispase had no effect on the α subunit of PH-30 (Fig. 4 B, lanes 2-5).

Treatment of testicular cells with proteinase K led to the disappearance of the pre α band and reduced (by ~ 10 kD) the molecular weight of > 50% of the pre β molecules detected on immunoblots. Treatment of testicular cells with chymotrypsin or trypsin, however, had no effect on pre α or pre β (not shown). These data indicate that, on testicular cells, pre α and, at least the majority of, pre β are accessible to external protease. However, pre β found on testicular cells (as opposed to that on testicular sperm) could not be converted by *in vitro* proteolysis to species that migrate in the vicinity of the β bands found on mature epididymal sperm. Correct processing of pre α to α may therefore be a prerequisite for correct processing of pre β.

Protease treatment of testicular sperm exposes epitopes on the PH-30 protein

Immunofluorescence experiments have revealed that neither the fusion inhibitory monoclonal antibody (mAb PH-30) nor the noninhibitory control monoclonal antibody (mAb PH-1) react with native PH-30 on testicular sperm whereas both monoclonal

antibodies react with native PH-30 on mature epididymal sperm [5]. The polyclonal antibody raised against PH-30 protein does bind to live untreated testicular sperm ([48], and see below). We therefore tested whether treatment of testicular sperm with proteases that cleave pre β *in vitro* (proteinase K, chymotrypsin, trypsin, and dispase, see Fig. 3) unmask the epitopes seen by mAb PH-30 and mAb PH-1.

Fig. 5 shows the results of a typical experiment in which testicular sperm were treated with trypsin on ice. Immunofluorescence photographs were taken of testicular sperm before trypsinization (panels A,C) and after trypsinization for 1 h on ice (panels B,D, 100 μg/ml trypsin). Panels A and B were stained with the polyclonal PH-30 antiserum, whereas panels C and D were stained with the monoclonal antibody mAB PH-30. Staining with the polyclonal antibody clearly revealed the presence of PH-30 on the whole head of untreated (panel A) and on trypsin-treated (panel B) testicular sperm. The staining pattern with mAb PH-30 was different. As shown previously [5, 48] mAB PH-30 does not recognize untreated testicular sperm (panel C). However, after trypsinization mAb PH-30 bound to testicular sperm (panel D). The staining pattern shown is representative of virtually all of the trypsin-treated testicular sperm analyzed. The staining pattern in panel D resembled that seen with the polyclonal antibody (panel B) in that staining was seen over the whole head. At present we cannot explain why the monoclonal antibody gave a punctate staining (Fig. 5D) while the polyclonal antibodies gave a more diffuse staining pattern (Fig. 5B).

Results obtained with the other proteases and with both mAB PH-30 and mAB PH-1 are shown in Table I. Whereas the polyclonal antibody bound to PH-30 on testicular sperm, neither mAB PH-30 nor mAB PH-1 bound to testicular sperm. However, treatment of testicular sperm with either proteinase K, chymotrypsin, trypsin or dispase (on ice) allowed binding of both mAb PH-30 and mAb PH-1. As described above (Fig 5), in all cases following protease treatment staining was seen over the whole sperm head. Staining with both monoclonal antibodies was punctate in appearance and visible on virtually all protease treated testicular sperm. The staining intensity was roughly the same in all samples, with the exception of dispase-treated testicular sperm, which were stained relatively less intensely with mAB PH-30. Collectively these findings suggest that *in vitro* treatment of testicular sperm with several proteases leads to processing of pre β. Both *in vivo* and *in vitro*, processing unmasks at least two new epitopes on PH-30. One of the newly exposed epitopes appears to be important for sperm-egg fusion [5].

DISCUSSION

The fusion reactions that occur between enveloped viruses and their host cells are exoplasmic events. Although viral membrane fusion proteins are well characterized [3, 5, 7], very little is known about proteins mediating exoplasmic fusion events that occur between cellular membranes [1]. In this paper we have further characterized PH-30, a protein that has been shown previously to play a role in the exoplasmic fusion event that occurs between the plasma membranes of guinea pig sperm and egg [5].

On mature epididymal sperm, PH-30 exists as a tight complex of two immunologically distinct subunits, α and β. Affinity-purified polyclonal antibodies against the α subunit react only with α. Affinity-purified polyclonal antibodies and two monoclonal antibodies, that we show here to be directed against the β subunit, react only with β. These findings confirm earlier results showing different polypeptide patterns following digestion of α and β with V8 protease [5]. Analysis with several antibodies directed against the β subunit indicates that there are at least three different forms of β (β_1, β_2 and β_3) on mature epididymal sperm. The functional significance of the multiple β bands is not yet apparent. The PH-30 α and β chains form a complex that is resistant to treatment with SDS. This finding explains the ability of monoclonal antibodies directed against the β subunit to coimmunoprecipitate both α and β [5]. The PH-30 α/β complex found on mature epididymal sperm behaves as an integral membrane glycoprotein. It is not extracted from the

sperm membrane with high salt or alkali, it binds quantitatively to Con-A Sepharose, and the apparent molecular weights of both of its subunits are reduced upon treatment with endoglycosidase F.

Both subunits of PH-30 are made as larger precursors, pre α and pre β. Pre α and pre β are processed at different developmental stages and complexes of α and β exist throughout sperm maturation. The susceptibility of pre α and the majority of pre β to proteinase K indicates that both precursors are present on the surface of testicular cells. By the time the cells have matured to become testicular sperm, pre α has been processed to its mature size and does not undergo further detectable proteolytic modifications. Pre β, on the other hand, is present on the surface of both immature testicular cells as well as testicular sperm. Proteolytic processing of pre β occurs during passage through the epididymis and appears to occur in at least two steps. At an early stage of epididymal maturation (in the distal corpus epididymis), two bands of decreased molecular mass are seen, pre-β^* and a band that comigrates with $\beta_{1,2}$ of mature epididymal sperm. Final processing of pre β, which leads to the appearance of β_3, occurs in the proximal cauda epididymis.

Processing of PH-30 thus appears to be under tight developmental regulation. In rodents, sperm become fully fertilization competent when they reach the proximal cauda epididymis. Acquisition of fertilization competence is thought to be due to biochemical alterations of sperm surface proteins occurring during epididymal passage [49]. Our results and those presented by Phelps et al. [48]

strongly suggest that proteolysis plays an active role in the maturation of functionally important sperm surface proteins. The final processing step that we observe for the β subunit of PH-30 occurs in, or in transit to, the proximal cauda epididymis. Coincident with this cleavage, the epitope recognized by the fusion-inhibitory monoclonal antibody is revealed. Concomitantly, PH-30 localizes [48] to the posterior head, the region where fusion with the egg is thought to be initiated and/or continued [5, 10], and sperm become fertilization competent. Therefore, it is tempting to speculate that the final processing event that generates β_3 on epididymal sperm may activate PH-30 and may therefore be responsible, at least in part, for the acquisition of sperm fertilization competence.

Interestingly, we found that *in vitro* proteolysis of live testicular sperm with chymotrypsin, trypsin or dispase cleaves pre β into distinct polypeptides of apparent molecular weights similar to those of $\beta_{1,2}$ and β_3 found on mature epididymal sperm. An attractive interpretation of these results is that the β subunit of PH-30 on testicular sperm may have two domains, a precursor domain that is sensitive to proteolysis and a second, perhaps functional, domain that is resistant to further proteolysis. Given that sperm may be exposed to a proteolytic environment in the epididymis and again following the acrosome reaction, it is not surprising that mature PH-30, which functions in fusion with the egg plasma membrane appears to be, at least relatively, protease resistant.

The monoclonal antibodies mAb PH-30 (which inhibits fusion) and mAb PH-1 (which does not inhibit fusion) do not bind to testicular sperm [5]. However, treatment of testicular sperm with either proteinase K, chymotrypsin, trypsin, or dispase exposes the sites reactive with both monoclonal antibodies. Exposure of these epitopes could be due to removal of the precursor domain of pre β, to more complex conformational changes in the structure of β, and/or to modification or removal of the glycocalyx that covers the sperm surface [50]. In this respect it is interesting that trypsinization of testicular sperm exposes an epitope on at least one other surface protein, AH-40 [48].

In the *in vitro* proteolysis experiments presented here, testicular sperm were treated with high concentrations of protease (100 μg/ml trypsin, chymotrypsin, proteinase K; 500 μg/ml dispase) for 1 h on ice. Under these conditions, the newly exposed epitopes recognized by mAbs PH-30 and PH-1 were found over the entire sperm head. Conversely, when testicular sperm were treated with a 5-fold lower concentration of trypsin (20 μg/ml) for 5 min at RT followed by a 1h incubation at RT, the epitope recognized by mAb PH-30 was found only in the posterior head region [48], where it is localized on mature epididymal sperm [5]. The explanation for this difference requires further experimentation. Localization may be impeded at low temperature due to interference with an energy dependant process or to reduced lateral diffusion of PH-30. Alternatively, the harsher proteolysis conditions used here may have destroyed an auxiliary protein(s) involved in the localization process. In any event, the data

shown here are consistent with the observation that exposure of the epitopes recognized by mAb PH-30 and mAb PH-1 precedes localization of PH-30 to the posterior head [48]. PH-30 is present at high surface density ($\geq 5 \times 10^3$ α/β dimers per μm^2) in the posterior head of mature sperm (Primakoff, unpublished). Localization of PH-30 may therefore help establish a critical concentration required for its function.

Since PH-30 is involved in an exoplasmic fusion event, we asked at the outset whether PH-30 shares features with viral membrane fusion proteins. Our results show that PH-30 does display properties similar to those of viral membrane fusion proteins. It is an integral membrane glycoprotein, it is composed of two tightly associated subunits, and both subunits are made as precursors. Strictly speaking, the processing of PH-30 from two separate precursors is more complex than that of any simple viral fusion protein analyzed to date. Furthermore, we are fully aware that the similarities of PH-30 and viral fusion proteins may be completely coincidental as these features are shared by other cell surface proteins. However, the basic biochemical features of PH-30 established here are consistent with what is known about exoplasmic membrane fusion proteins. Moreover, the finding that the PH-30 subunits are made as precursors suggests an important developmental regulation of PH-30 function. The correlation between the processing of the β subunit of PH-30 and the acquisition of fertilization competence strengthens the idea that PH-30 plays an important role in the fusion event between sperm and egg.

ACKNOWLEDGEMENTS

The authors acknowledge Tyra Wolfsberg for helpful discussion, Samuel Green, Barry Gumbiner and Adam Linstedt for critical reading of the manuscript, Jaquie Lopez for Fig. 2B, and Joanne Tredick-Kline and Laura Woolman for excellent technical assistance.

Figure 1. PH-30 is an SDS-resistant complex of two immunologically distinct proteins. Immunoblot analysis of nonreduced epididymal sperm extracts that were either boiled in 2% SDS (Panel A) or treated at RT with 0.5% SDS (Panel B) prior to electrophoresis. The primary antibodies used in panel A were: rabbit PH-30 polyclonal antiserum (lane 1), affinity-purified α specific antibodies (lane 2), affinity-purified β specific antibodies (lane 3), mouse monoclonal antibodies mAB PH-30 (lane 4) and mAB PH-1 (lane 5). Lanes 6 and 7 were probed with affinity-purified β specific antibodies and represent two fractions of an immunodepletion experiment: PH-30 protein that does not bind to mAB PH-30 (lane 6) and PH-30 protein that does bind to mAB PH-30 (lane 7). As controls, an epididymal sperm extract was probed with nonspecific mouse IgG (lane 8) or with preimmune rabbit IgG (lane 9). The primary antibodies in panel B, lanes 1-3 were the same as in panel A, lanes 1-3; preimmune rabbit-antiserum was used as control in Panel B, lane 4.

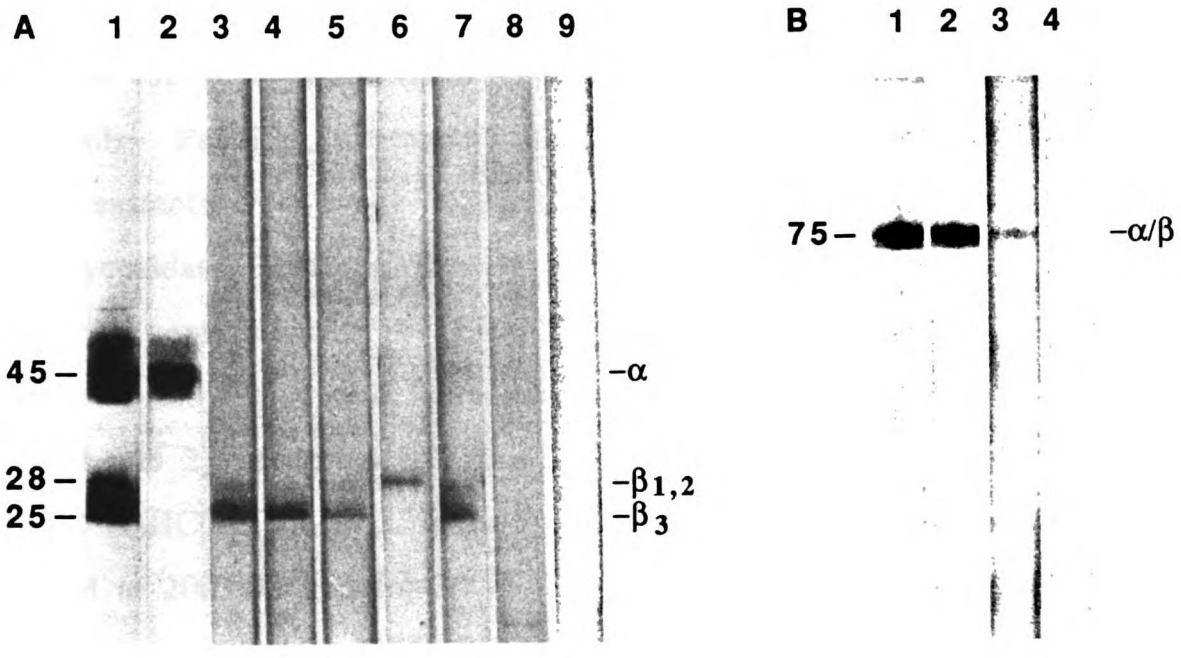

A
1 2 3 4 5 6 7 8 9
45 –
28 –
25 –
–α
–β1,2
–β3
B
1 2 3 4
75 –
–α/β

Figure 2. PH-30 is an integral membrane glycoprotein. All samples were run on nonreducing SDS-PAGE and immunoblotted with rabbit polyclonal anti-PH-30 antiserum. Panel A, binding of PH-30 from mature epididymal sperm extracts to Con-A Sepharose CL-4B. Material bound (lane 1) or in the supernatant (lane 2) after incubation with Con-A Sepharose CL-4B beads. Material bound (lane 3) or in the supernatant (lane 4) after incubation with Sepharose CL-4B beads. Panel B, treatment with endoglycosidase F. Epididymal sperm extracts were mock digested (lane 1) or digested (lane 2) with endoglycosidase F as described in the Methods section. Panel C, membrane extractability of PH-30 from mature epididymal sperm. Mature epididymal sperm were treated with high salt (0.5 M KCl, lanes 1 and 2), moderate salt (0.1 M KCl, lanes 3 and 4) or with alkali (0.1 M NaHCO3, pH 11.5, lanes 5 and 6) for 30 min on ice and then pelleted at 200,000 x g for 30 min in a TLA 100.3 rotor. Equal volumes of pellets (lanes 1, 3 and 5) and supernatants (lanes 2, 4 and 6) were analyzed for PH-30 protein as described above.

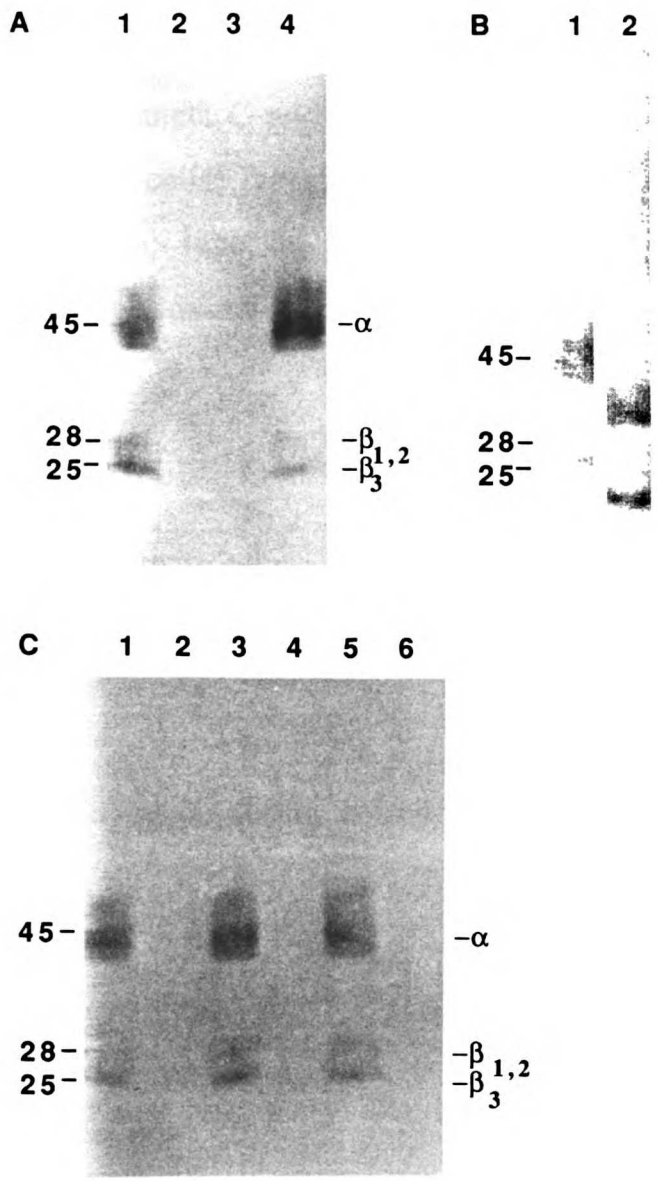

A
1 2 3 4
45-
28-
25-
-α
-β1,2
-β3
B
1 2
45-
28-
25-
C
1 2 3 4 5 6
45-
28-
25-
-α
-β1,2
-β3

Figure 3. Both subunits of PH-30 are made as larger precursors. Cell extracts from sperm at different developmental stages were run on non-reducing SDS-PAGE and immunoblotted with affinity-purified α specific antibodies (panels A and C) and affinity-purified β specific antibodies (panels B and D). Extracts in panels A and B were boiled in 2% SDS; extracts in panels C and D were treated at RT with 0.5% SDS. Lane 1, testicular cells; lane 2, testicular sperm; lane 3, distal corpus epididymal sperm; lane 4, proximal cauda epididymal sperm; lane 5, distal cauda epididymal sperm.

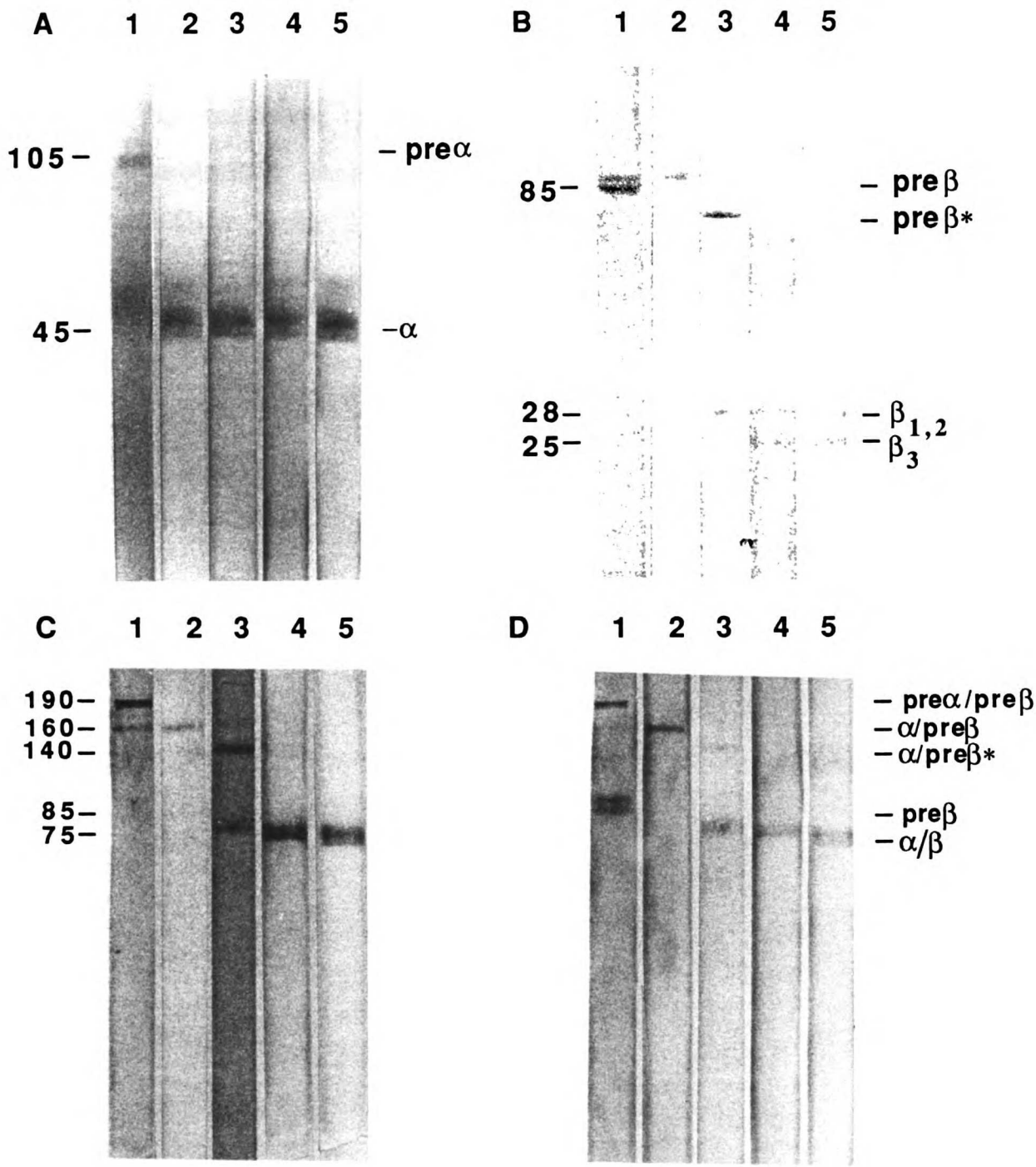

A
1 2 3 4 5
105–
45–
– preα
–α
B
1 2 3 4 5
85–
28–
25–
– preβ
– preβ*
– β1,2
– β3
C
1 2 3 4 5
190–
160–
140–
85–
75–
D
1 2 3 4 5
– preα/preβ
–α/preβ
–α/preβ*
– preβ
–α/β

Figure 4. Proteolytic processing of PH-30 on testicular sperm in vitro. Extracts of testicular sperm or distal cauda epididymal sperm were boiled in 2% SDS, run on non-reducing SDS-PAGE and probed with β specific (panel A) or α specific antibodies (panel B). Testicular sperm were mock digested (lane 1) or treated with 20 μg/ml proteinase K (lane 2), chymotrypsin (lane 3), TPCK-treated trypsin (lane 4), or 100 μg/ml dispase (lane 5) for 2 h at 37°C. An extract of untreated distal cauda epididymal sperm is shown in lane 6.

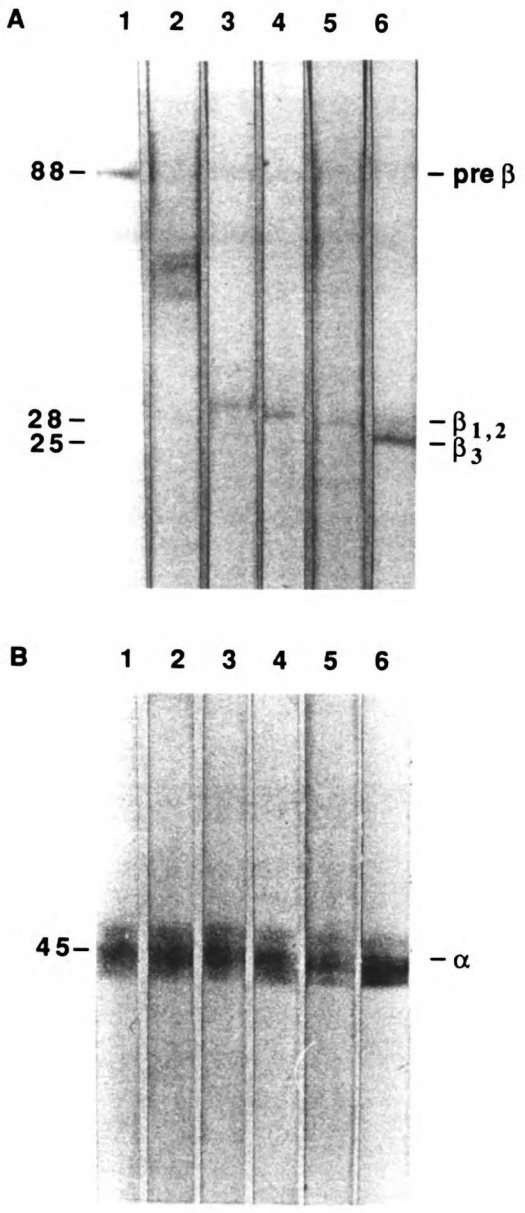

A
1 2 3 4 5 6
88–
– pre β
28–
25–
– β1,2
– β3
B
1 2 3 4 5 6
45–
– α

Figure 5. In vitro trypsinization of testicular sperm unmasks the mAB PH-30 epitope. Immunofluorescence micrographs of mock digested testicular sperm (A,C), and testicular sperm treated with 100 μg/ml TPCK-treated trypsin for 1 h on ice (B,D). Samples in panels A and B were stained with the rabbit PH-30 polyclonal antiserum; samples in panels C and D were stained with mAB PH-30. Cells shown are representative of the vast majority of the cells viewed. Primary antibodies were added to live cells on ice. Bar =2 μm

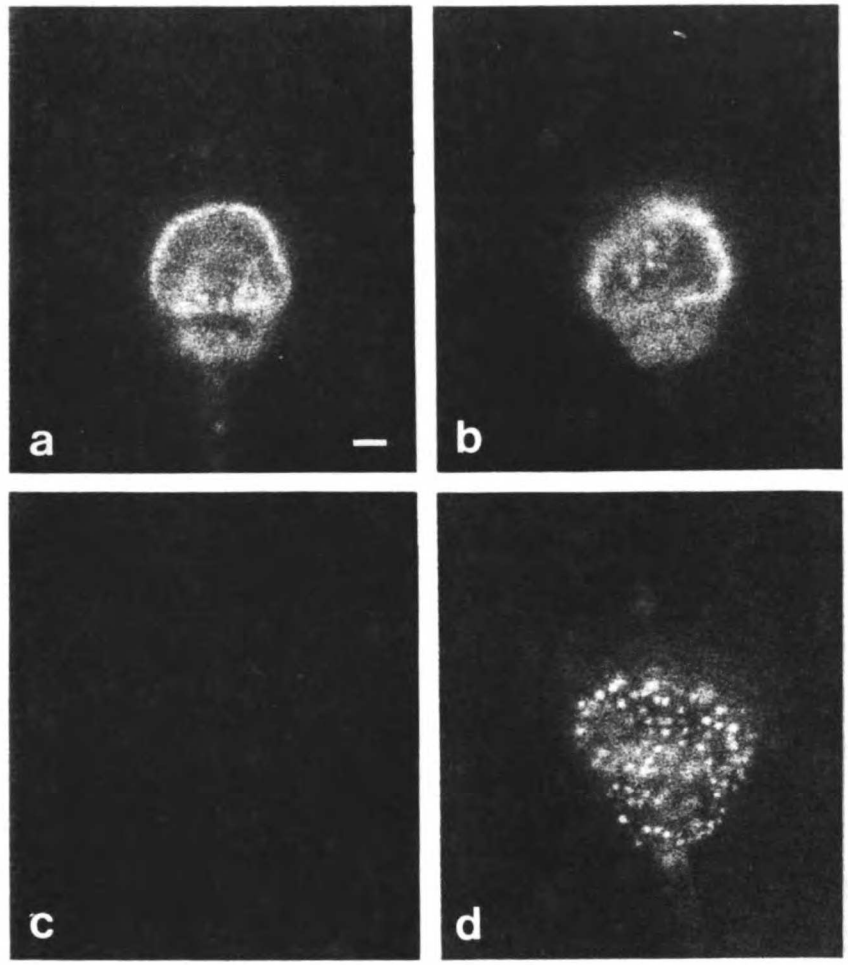

a
b
c
d

TABLE I Binding of the monoclonal antibodies mAb PH-30 and mAb PH-1 to testicular sperm following treatment with different proteases

Protease	Immunofluorescence	
	mAB PH-30	mAB PH-1
None (mock digestion)	-	-
Proteinase K	++	++
Chymotrypsin	++	++
Trypsin	++	++
Dispase	+	++

Testicular sperm were either mock digested or treated with 100 μg/ml of proteinase K, chymotrypsin, or trypsin, or with 500 μg/ml dispase for 1 h on ice. Samples were stained with the indicated antibodies as described in the Methods section. The results were the same in three separate experiments. A minus indicates no staining. A double plus indicates strong, and a single plus indicates weaker staining. In all positive samples (+, ++), punctate staining was seen over the whole head of virtually all sperm cells.

CHAPTER 4

Cloning of cDNAs encoding the mature subunits of PH-30 α and ß

ABSTRACT

The guinea pig sperm protein PH-30 is thought to play a role in sperm-egg membrane fusion mainly because this process is inhibited by one, but not by a second anti PH-30 monoclonal antibody [5]. Additional biochemical characterization of PH-30 revealed properties consistent with PH-30 being a membrane fusion protein (chapter 3). This chapter describes the cloning and sequencing of cDNAs encoding the mature α and ß subunits of PH-30. A cDNA encoding the mature α subunit of PH-30 was cloned (by Tyra Wolfsberg) in a nested PCR strategy followed by the RACE protocol. A fragment of the gene encoding the ß subunit of PH-30 was obtained using a nested PCR strategy, and was used to obtain a cDNA encoding for mature PH-30 ß from a lambda gt 11 library made from guinea pig testicular cells.

Mature PH-30 ß was found to be a class I integral membrane glycoprotein. Its N-terminal domain was found to be highly homologous to a family of RGD containing snake venom peptides that competitively antagonize platelet aggregation by inhibiting fibrinogen binding to the platelet integrin gpIIb/IIIa. We therefore propose that PH-30 ß binds to an integrin on the egg plasma membrane. PH-30 ß would thus mediate tight binding of the posterior head of sperm to the egg plasma membrane, a prerequisite for membrane fusion. The involvement of a gpIIb/IIIa type integrin might explain the calcium dependence of sperm egg binding/fusion, and could be responsible for signaling events that accompany fusion.

The mature α subunit of PH-30 was also found to be a class I integral membrane glycoprotein. In addition to its transmembrane domain it contains a hydrophobic domain with features similar to viral fusion peptides. A sequence encompassing the putative fusion peptide of PH-30 α was found to have a high degree of similarity to a sequence in the E1 subunit of the rubella virus fusion protein.

Thus like many viral fusion proteins PH-30 is predicted to be a complex of two class I integral membrane glycoproteins, one (ß) posessing binding activity, and the other (α) possessing fusion activity. These findings lend strong support to the proposal that PH-30 is a sperm-egg fusion protein.

INTRODUCTION

The proteins that mediate fusion between viruses and their host cells are well characterized (reviewed in [3]). In contrast, proteins that mediate cellular membrane fusion reactions remain ill defined. A protein located on the surface of the posterior head of guinea pig sperm, PH-30, is thought to play a role in sperm egg fusion based on the inhibitory action of an anti PH-30 monoclonal antibody [5]. Subsequent biochemical characterization of PH-30 revealed that it shares feature in common with viral fusion proteins (chapter 3). It is an integral membrane complex of two glycoproteins that are both made as larger precursors in the testis. Processing of the ß subunit of PH-30 coincides with the acquisition of fertilization competence. In this chapter I report the results of cloning and sequencing cDNAs encoding both subunits of PH-30. The results indicate that PH-30 is a complex of one subunit with a possible role in sperm-egg binding, and a second subunit with a possible direct role in membrane fusion. The predicted topology of the complex resembles many viral membrane fusion proteins.

RESULTS

Generation of protein sequence information for the α and ß subunits of PH-30

PH-30 was purified by affinity chromatography on a column containing the monoclonal Ab PH-30 as described previously [5] with modifications as described (Materials and Methods). Sperm obtained from the guinea pig distal cauda epididymis was used to generate the protein sequence information used to clone the cDNAs encoding the α and ß subunits.

Purified PH-30 protein was reduced, subjected to SDS-PAGE and transferred to an immobilon membrane. Bands corresponding to α and ß were visualized with Coomassie Blue stain, excised, and subjected to automated Edman degradation as described in the Materials and Methods section. In this manner, N-terminal protein sequence information for α and ß, 18 amino acids and 7 amino acids, respectively, was obtained.

To obtain additional sequence information, affinity purified PH-30 was subjected to SDS-PAGE (under nonreducing conditions) and the bands corresponding to PH-30 α and ß were cut out of the gel, electroeluted, and further purified and concentrated as described in the Materials and Methods section. Samples of the α (lane 2) and ß (lane 3) subunits prepared in this manner are shown in Fig. 1.

Purified α subunit was trypsinized, reduced, alkylated, and subjected to reverse phase HPLC (see Materials and Methods for details). The sequence (14 amino acids) of a major tryptic fragment was then determined (see table I). Purified ß-subunit was digested with CNBr or V8 protease, subjected to SDS-PAGE, transferred to an immobilon membrane, and sequenced as described in the Materials and Methods section. The sequences obtained from the resulting 4 bands are shown in table 1.

To obtain additional protein sequence information from the ß subunit, protein electroeluted from a preparative SDS gel was boiled in 2% SDS, 25 mM DTT for 10 min, and alkylated with 50 mM IAA for 30 min at room temperature. We found it necessary to use these more stringent than standard (ref) reduction conditions in order to obtain longer stretches of peptide sequence. This presumably reflects the highly disulfide-bonded nature [5] of PH-30 ß. Following removal of SDS, DTT and IAA by acetone precipitation, the protein was cleaved to completion with CNBr (assayed on a minigel), concentrated, dissolved in 8 M urea and ammonium bicarbonate, and trypsinized (1:10 enzyme:protein ratio). The resulting peptide fragments were separated by HPLC and subjected to protein sequencing (see Materials and Methods for details). A summary of all protein sequences found to date, and the respective methods used to obtain them, is shown in Table 1. One of the ß sequences, an 8 amino acid tryptic fragment, was obtained twice, and another sequence corresponding to the N-terminus of mature ß, was seen 4 times.

Cloning of a cDNA encoding mature PH-30 α (performed by Tyra Wolfsberg)

The two protein sequences obtained for PH-30 α (Table 1) were used to design oligonucleotide primers for use in a PCR. The N-terminal protein sequence was used to design two degenerate nonoverlapping ("nested") primers in the 5' - 3' direction of the coding sequence. The second, internal amino acid sequence was similarly used to design two degenerate nested primers in the 5' - 3' direction of the noncoding sequence. RNA isolated from whole guinea pig testis was used to prepare oligo dT primed cDNA which in turn was used as a template for PCR. All four possible pairs of sense and antisense primers were used. In each case a band of around 300 bp was seen on an ethidiumbromide stained agarose gel. This 300 bp DNA fragment was subcloned into a bluescript plasmid vector and sequenced. An open reading frame was found that encoded the N-terminal and tryptic peptide sequences determined by automated Edman degradation. This 300 bp segment therefore encoded the N-terminus of mature α through the C- terminus of the tryptic fragment. The RACE procedure (rapid amplification of cDNA ends, [51]) was then used to clone the 3' end of the α gene. Briefly, a 57 nucleotide primer, with a 3' oligo dT sequence and a known (17 bp) specific 5' sequence extension, was used to prime the synthesis of cDNA from testicular mRNA by reverse transcription. The resulting cDNA was then used in a PCR with a perfectly matched 17 nucleotide primer corresponding to the α sequence, and a second primer corresponding to the known 17 nucleotide 5' sequence extension of

the 57 nucleotide oligo dT containing primer. The candidate PCR amplified DNA sequence (1117 bp) was then subcloned into a bluescript vector and sequenced. The presence of a polyadenylation site followed by a poly A tail indicated that the product encoded the 3' end of the α gene.

The mature α cDNA sequence, and the deduced protein sequence is shown in fig 2. It is predicted to encode a protein of MW 30 kD with single transmembrane domain, an internal stretch of hydrophobic amino acids (fig 3), and one N-linked glycosylation site. The internal hydrophobic stretch can be modeled as a sided α helix with bulky hydrophobic residues on one face and small uncharged amino acids on the other (not shown). Because the protein undergoes extracellular processing (chapter 3), we infer that it is a class I integral membrane glycoprotein (i.e. its N-terminus is outside and its C-terminus inside the sperm plasma membrane). A northern blot of guinea pig testis RNA probed with the original 300 bp PCR fragment showed a band of about 3 kb. The molecular weight of the mature α subunit is about 60 kD when boiled and reduced and about 45 kD when boiled but not reduced. The molecular weight of pre α is about 105 kD. The full length clone for pre-α is therefore expected to be around 3 kb. The apparent discrepancy between the molecular weight of mature α deduced from the nucleotide sequence, and that seen on SDS gels can be only partially explained by glycosylation. We assume that other posttranslational modifications might contribute to the discrepancy. The nucleotide sequence encoding the precursor domain of the α subunit of PH-30 is currently being determined.

Cloning of a cDNA encoding the mature ß subunit of PH-30

Generation of a high stringency probe for PH-30 ß by nested PCR

The 28 amino-acid N-terminal sequence of PH-30 ß (Table 1) was used to design two primers that were nested in the sense orientation starting from the N-terminus. Two more nested primers corresponding to the 22 amino acid internal tryptic peptide (see table) were therefore designed in the antisense orientation. A primary PCR was performed using the respective 5' (outer) primers. The reaction mixture was then diluted 1/400 and used as a template for a second PCR with all combinations of the 3' (inner) and 5' (outer) primers. When the resulting DNA fragments were run on an agarose gel, a major band of 230 bp was seen in the reaction with the 5' (outer) N-terminal primer and the 3' (inner) tryptic fragment primer. An appropriately shorter band of 210 bp was observed when a combination of both 3' (inner) primers was used. The 230 and 210 bp bands were therefore considered candidate fragments the gene encoding PH-30 ß.

To confirm the identity of the 230 bp band, it was amplified in a preparative PCR, purified on a 5% acrylamide gel, and subjected to direct sequencing as described in the Materials and Methods section. The nucleotide sequence was found to encode for 13 amino acid of the N-terminal sequence as determined by automated Edman

degradation (these amino acids were not ones encoded by the primer sequence).

Identification and sequence of a lambda gt 11 cDNA clone encoding mature PH-30 ß

The 230 bp fragment of the PH-30 ß gene described above was labeled with ^{32}P using a random primer labeling kit (BRL) and used at high stringency to probe a lambda gt 11 library made from guinea pig testis mRNA (gift of Dr. Paul Primakoff). Six potentially positive clones were found, but only one of these was shown, by partial sequencing, to encode for PH-30 ß sequences. This latter clone was then sequenced using the technique for directly sequencing PCR fragments in combination with "primer walk" as described in the Materials and Methods section. Directly sequencing PCR bands does not lead to an increased sequencing error rate because a population of DNA molecules generated from a large number of identical templates was sequenced in every reaction. Any error made by the taq polymerase during amplification would therefore only affect a very small fraction of the template molecules.

After the sequence of the lambda clone was determined by direct sequencing, it was confirmed by resequencing the entire coding region of mature ß in both orientations using template generated with PCR from first strand testicular cDNA. The cDNA sequence obtained (fig 4) encoded the mature ß protein and three additional

upstream amino acids of pre-ß. All of the peptide sequences that we had obtained (Table 1) were found in the deduced amino acid sequence, confirming the identity of the cDNA clone. These sequences are underlined in fig 5. The deduced amino acid sequence predicts a molecular weight of 39 kD for the mature ß subunit of PH-30 ß. Since PH-30 ß is glycosylated (see chapter 3), this value is consistent with the observed apparent molecular weight of 45 kD seen on SDS gels run under reducing conditions. A hydrophobicity analysis indicated that PH-30 ß has a single transmembrane domain and a short cytoplasmic tail (fig 6). As with the α subunit (see above), since pre ß is processed extracellularly (chapter 3), the mature ß subunit is predicted to be a class I integral membrane glycoprotein (fig 6). PH-30 ß is very cysteine rich (9.2 % cysteins), as predicted earlier by its significantly slower migration when analyzed on reducing compared with nonreducing SDS gels.

Genbank homology search

The genbank database was searched for sequences related to the deduced protein sequence of mature PH-30 ß. The 79 amino acid snake venom protein bitistatin [52] was found to share a high degree (53%) of sequence identity with PH-30 ß over its N-terminal 79 amino acids (see fig 7 for sequence alignments). The positions of the conserved amino acids (including conserved cystein residues) between PH-30 ß and bitistatin were identical starting with the N-terminal serine of mature ß. Bitistatin is a member of a highly conserved family of RGD containing snake venom proteins that inhibit platelet aggregation by competitively inhibiting the binding of fibrinogen to the platelet integrin (fibrinogen receptor) gpIIb/IIIa [53, 54]. The venom protein barbourin has a KGD instead of an RGD sequence and binds gpIIb/IIIa with high specificity [55]. Bitistatin and related venom platelet aggregation inhibitors are the strongest competitive antagonists known; they bind the GP IIb/IIIa receptor better than fibrinogen. PH-30 also shares homology with a number of other proteins that are integrin ligands, such as mouse and human laminin B2 [56], tenascin [57] and S-laminin [58]. In the position where the snake venom proteins have the sequence R (or K) GD, PH-30 ß has the sequence RESTDEC. Interestingly, the mouse laminin B2 protein, which can be aligned with PH-30 ß and the snake venom proteins (trigramin as an example in fig 7), contains a very similar sequence, RGSTDEC in a putative cell binding domain (region V, [56, 58, 59]). The regions of homology with the other proteins lie within the cysteine rich repeat domains. PH-30 ß also shows 20% identity over

260 amino-acids with the Drosophila protein notch [60] which is thought to play a role in cell-cell adhesion (and Drosophila development) via a receptor mediated process [61].

DISCUSSION

The mature α subunit of PH-30

The deduced amino acid sequence indicates that PH-30 α is a class I integral membrane glycoprotein with a short (44 amino acid) cytoplasmic tail. This is consistent with our biochemical evidence (chapter 3) that PH-30 behaves as an integral membrane glycoprotein. The protein sequence contains a number of cysteines capable of forming disulfide bonds. This feature was predicted based on the considerably slower migration of α on SDS gels after reduction and carboxymethylation (a shift in apparent molecular weight from 45 kD to 60 kD [5]). The sequence of PH-30 α indicates one potential N-linked glycosylation site, consistent with the results of endoglycosidase F treatment of PH-30 (chapter 3). The predicted molecular weight of mature α deduced from the protein sequence is about half and two thirds of those seen when α is run on SDS gels under reducing and non-reducing conditions, respectively. Even though glycosylation might account for part of this discrepancy, other unknown modifications might be present. Membrane proteins frequently migrate more slowly than expected on SDS polyacrylamide gels.

In addition to the transmembrane domain, the PH-30 α sequence predicts a second hydrophobic domain between residues 89 and 112 (fig 3). This sequence can be modeled as a sided α helix, a feature common to all viral fusion peptides [3]. In a sequence homology

search, the residues 82-97 of PH-30 α (which encompass the amino terminal half of the putative fusion peptide of α) showed a strong homology to a region of the E2 glycoprotein of rubella virus. This homology falls within one of the two putative fusion peptides of rubella virus [62]. Over a stretch of 16 amino acids, 13 were either identical or highly conserved (fig 8). In a model of the domain structure of mature α (fig 9, as drawn by Fernando Bazan, Fletterik lab), the conserved sequence is predicted to lie in a hinge region between two cysteine rich domains. The significance of this homology remains to be determined. Aside from the possibility of pure coincidence, it could have arisen from convergent evolution or from true conservation. The latter possibility would suggest that the viral fusion function derived from a cellular fusion function. The sizes of PH-30 α and the E2 glycoprotein of rubella virus as well as the distances between their putative fusion peptides from the transmembrane domains are, however, quite similar.

The mature ß subunit of PH-30

The ß subunit of PH-30 shares significant homology with bitistatin [52], a member of a family of short (60-80 amino acid) RGD sequence containing snake venom peptides. Bitistatin and related venom proteins competitively inhibit the binding of fibrinogen to the platelet integrin GP IIb/IIIa [53, 54]. PH-30 ß also shares homology with other integrin ligands such as tenascin [57] and S-laminin [58]. Before fusion occurs, sperm must bind to the egg plasma membrane (reviewed in [63]). The deduced amino acid sequence of the ß subunit

of PH-30 suggests that it plays at least a role in this interaction. The similarity of PH-30 ß with integrin ligands further suggests that the receptor on the egg plasma membrane is a member of the integrin family [64].

Although previous studies have suggested that a sperm receptor exists on the egg plasma membrane [36], the molecules responsible for this interaction have not yet been identified. Proteolysis of zona free mouse eggs blocks their ability to fuse with sperm [36]. Specific mouse egg surface proteins that are susceptible to proteolysis have been identified as candidate sperm receptors [65]. In other systems, such as guinea pig, however, sperm-egg fusion is not very sensitive to proteolysis [10]. Recently, RGD containing peptides were found to inhibit fusion of human or hamster sperm with zona free hamster eggs, indicating that an integrin might be involved in this process [66]

Even though PH-30 ß does not contain an RGD sequence, the high degree of homology between the N-terminal 80 amino acids of PH-30 ß and the RGD containing snake venom proteins strongly suggests that it does belong to the same family of proteins. In the sequence alignment (fig 7) PH-30 ß contains the sequence RESTDEC where the snake venom peptides have an RGD or in one case, a KGD (barbourin, [55]). Barbourin apparently has exclusive specificity for gpIIb/III binding, whereas RGD containing venom peptides do not. Given the alignment of the sequence RESTDEC with the RGD sequence in the venom peptides, it is tempting to speculate that this sequence might be a specific binding sequence for guinea pig eggs. We are presently

investigating the role of the RESTDEC sequence as a candidate for mediating sperm-egg binding. However, a different additional sequence or domain of PH-30 ß could also be responsible for its binding function.

When the genbank database was searched with the RESTDEC peptide sequence alone, mouse laminin B2 was found to have a similar sequence, RGSTDEC (fig 7), in domain V, a cysteine rich region involved in cell attachment [56, 59]. The alignment between PH-30 ß and laminin B2 can be extended to adjacent sequences, and laminin B2 can be aligned with the snake venom peptides as well (trigramin [67] is shown as an example in fig 7). Laminin is an integrin ligand consisting of three polypeptide chains, laminin A, B1 and B2 [59]. Laminin A contains an RGD sequence, but both laminin B1 and B2 do not, even though the laminin molecule is thought to have several receptor binding sites. A region on laminin B1 with the sequence YIGSR has been implicated in cell attachment [68], but the binding domain on laminin B2 has not yet been found. It will be interesting to see if the RGSTDEC sequence represents a laminin B2 binding sequence.

Sperm-egg fusion requires Ca++ [10]. Some integrins require Ca++ for proper function and the gpIIb/IIIa complex is known to dissociate when Ca++ is removed [54]. One might therefore speculate that the Ca++ requirement for sperm-egg fusion is due, at least in part, to the role of a Ca++ dependent integrin.

Integrins are further implicated in signalling processes [54]. Sperm-egg fusion signals cortical granule exocytosis and the prevention of polyspermy [10, 69]. It is therefore possible that binding of PH-30 to an integrin on the egg may trigger cortical granule exocytosis and/or the prevention of polyspermy.

The complex of PH-30 α and ß

The finding that the PH-30 ß subunit is likely to provide adhesive properties adds to the features PH-30 has in common with known viral membrane fusion proteins, most of which display a binding and a fusion activity [3, 7]. In the case of influenza HA, binding and fusion are accomplished by the HA protein, which binds sialic acid residues on the target membrane and fuses in response to a drop in pH [3, 7]. For HIV, the fusion protein GP40/120 binds to the CD4 receptor on the target cell which is thought to trigger the fusion process [1]. In analogy to viral fusion proteins, ß would bind a putative egg PH-30 receptor and bring the posterior head of the sperm close enough to the egg plasma membrane to allow fusion. As mentioned above, the PH-30 receptor is most likely a member of the integrin receptor family [64].

Viral fusion proteins with two subunits (e. g. those of arbo-, corona-, and bunyaviruses) are complexes of two class I glycoproteins. Rubella virus is an arbovirus and hence its fusion protein, which shares a short stretch of sequence similarity to PH-30 α, is a member

of this group. For this class of viral fusion proteins one of the subunits is thought to have binding activity, and the second is thought to have fusion activity. The complex of PH-30 α and ß subunits thus has the same topology as the group of viral fusion proteins and we predict that one subunit (ß) is involved in binding and the second subunit (α) is involved in fusion. Future studies will address whether the α and ß subunits serve these postulated functions.

Summary of results to date

In summary, our main new hypothesis regarding the role of PH-30 in sperm-egg fusion is that the fusion process is initiated by tight binding of PH-30 ß to a putative egg integrin (PH-30 receptor). This binding is followed by fusion of the sperm and egg plasma membranes, perhaps promoted by a fusion peptide present on PH-30 α. The involvement of integrins in sperm-egg binding and/or fusion might explain the Ca++ dependence of this process, and may be involved in signalling events such as cortical granule exocytosis.

Perspective and experimental proposals

One of the predictions resulting from the homology of PH-30 to bitistatin is that a PH-30 receptor will be present on the egg plasma membrane. Evidence for this receptor might be obtained directly or indirectly by a number of means. Direct evidence would be provided by crosslinking of PH-30 to an egg receptor, or purification of an egg

protein on immobilized PH-30. Other ligands such as the venom proteins, or specific peptides (such as RESTDEC or RGD) could also be used in immobilized form to purify the putative PH-30 receptor. Since mammalian eggs are not easy to obtain in large quantities, indirect evidence for a receptor could be provided by fusion inhibition with any of the above ligands. Further, one could monitor the binding of fluorescently labeled ligands to the egg (in the presence or absence of Ca++). Monoclonal or polyclonal antibodies against known integrins could also be employed, both in functional (binding and/or fusion) assays or in search of the egg receptor. Finally, the receptor could be cloned by homology with other integrins.

The results of cloning and sequencing the genes encoding the α and ß subunits of PH-30 further support the notion that it may be an analogue of viral membrane fusion proteins. Future experiments involving in vitro reconstitution of PH-30, transfection of PH-30 into foreign cells , and perhaps transgenic mice, will test whether PH-30 is necessary and sufficient to promote membrane fusion.

Table 1. Peptide sequences obtained from PH-30 α and ß by automated Edman degradation. Peptide sequence information for PH-30 α is shown in section A, and for PH-30 ß in section B. Peptide sequences are shown on the left side of the table in the one letter amino acid code, and the method used to prepare the respective peptide for sequencing is summarized on the right side of the table.

PH-30 α

Peptide sequence	Method of peptide purification
YETGQSGKTPLDTYKQDG	Immobilon blot of mature uncleaved α;(N-terminus)
ALFAAIQIPHGDD	Trypsin digest, HPLC

PH-30 ß

Peptide sequence	Method of peptide purification
SNPVVG	Immobilon blot of mature uncleaved ß; (N-terminus)
SNPVVGNNRVEQGEDCDCGSQEECQDTC	CNBr cleavage, trypsin digest, HPLC; (N-terminus)
STDECDLPEYCNGSSGACQEDL	CNBr cleavage, trypsin digest, HPLC
MGSVDGFEQLVTKN	V8 digest, Immobilon
MKIYAWISG	V8 digest, Immobilon
AQGASNWK	Trypsin digest, HPLC
SALE-VR	Trypsin digest, HPLC
ND(I/L) TV(N/S)---L	Trypsin digest, HPLC
VATV	Trypsin digest, HPLC
AQGP	CNBr cleavage, trypsin digest, HPLC
CPSWVCR	CNBr cleavage, trypsin digest, HPLC

Figure 1. Silver stained gel of affinity purified PH-30 protein. Lane 1 shows a silver stained gel of affinity purified boiled, but not reduced PH-30. Lane 2 and 3 show gel purified and electroeluted PH-30 α and ß, respectively.

1	2	3
PH-30	α	β

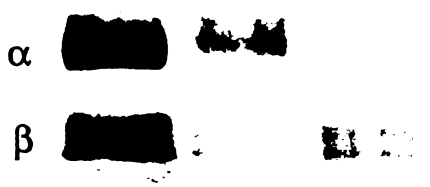


Figure 2. The DNA and amino acid sequence of mature PH-30 α. The cDNA encoding for mature PH-30 α was generated by the "RACE" procedure (see Materials and Methods), and was sequenced following subcloning into a bluescript plasmid vector. The entire nucleotide sequence, and the deduced protein sequence from an open reading frame that encoded for the two known α peptide sequences is shown in the three letter amino acid code. The polyadenylation site (AATAAA) is underlined. The twelve N-terminal amino acids were determined by automated Edman degradation of mature PH-30 α and were used to design oligonucleotide primers. The nondegenerate nucleotide sequence encoding for these amino acids has not yet been determined.

```
1/1                                                 31/11
                                                                ACA TAC AAA CAA GAT GGT ACC CCA
tyr glu thr gly gln ser gly lys thr pro leu asp thr tyr lys gln asp gly thr pro
61/21                                               91/31
TGT AAT GAG GGA TTC TTC TGT GTA AGT AAG GGG TGC ACG GAC CCT GGT ATT CAA TGC GCA
cys asn glu gly phe phe cys val ser lys gly cys thr asp pro gly ile gln cys ala
121/41                                              151/51
ACC TAT TTT GGG CAT GGT GCC AGG TCT GCC CCA GAT GCA TGT TAC ACT ACA TTG AAC AGC
thr tyr phe gly his gly ala arg ser ala pro asp ala cys tyr thr thr leu asn ser
181/61                                              211/71
ATA GGG AAT ATA TTT GGA AAC TGT GGT CAA TCA GGT AAT CCG ACC ACC TAT GTT GGG TGT
ile gly asn ile phe gly asn cys gly gln ser gly asn pro thr thr tyr val gly cys
241/81                                              271/91
TCA GGT GAT AGT ACA AAG TGT GGG AAA CTC ATA TGT ACA GGT ATT TCT TCA ATA CCT CCA
ser gly asp ser thr lys cys gly lys leu ile cys thr gly ile ser ser ile pro pro
301/101                                             331/111
ATC AGA GCT CTA TTT GCA GCG ATC CAG ATC CCT CAT GGA GAT GAC TGG TGC TGG AGC ATT
ile arg ala leu phe ala ala ile gln ile pro his gly asp asp trp cys trp ser ile
361/121                                             391/131
AGT AAC TTT GGG GAT CCT GCG TCC TCC CCC ACA GAA GGA GCT GTG TCA GCA GGC ACG TCT
ser asn phe gly asp pro ala ser ser pro thr glu gly ala val ser ala gly thr ser
421/141                                             451/151
TGC GCT TCA GGC AAA GCG TGT GTA AAT GCC CAG TGT TCT ACT TTC ACA CTT GAC ACT GCT
cys ala ser gly lys ala cys val asn ala gln cys ser thr phe thr leu asp thr ala
481/161                                             511/171
AAC TGT AGT GCA GCT GAA ATG TGT AAT GAG AAT GGA ATT TGC AAC AAT TTA GGG CAC TGC
asn cys ser ala ala glu met cys asn glu asn gly ile cys asn asn leu gly his cys
541/181                                             571/191
CAC TGT GGA GAT GGT TTT GCT CCC CCC AAC TGC AAA GAA CAA GGA ACT GGA GGT AGT ATA
his cys gly asp gly phe ala pro pro asn cys lys glu gln gly thr gly gly ser ile
601/201                                             631/211
GAT AGT GGT CCC CCT CCC CCT TCT AGT ACA CCT ACT GCA CCT CCT AAA CCA ACC CAA ACG
asp ser gly pro pro pro pro ser ser thr pro thr ala pro pro lys pro thr gln thr
661/221                                             691/231
ACA AAA GCA TCA AGT GAA AAC TTA GCA TTA ATT GGC CTG ATA ATT TTG GTA ATA CTA TTA
thr lys ala ser ser glu asn leu ala leu ile gly leu ile ile leu val ile leu leu
721/241                                             751/251
CTA CTT CTA GTT ATT TGT GCT ATC TGC CTT GGT ATA CCT GCT GAA GAA GCT CCT CCA CCA
leu leu leu val ile cys ala ile cys leu gly ile pro ala glu glu ala pro pro pro
781/261                                             811/271
CCA GAA GAG GAA GAA GCA GGG GAA CTG GAA GAA GAA CCA GAA CCA GAA CCA GAA CCG GAG
pro glu glu glu glu ala gly glu leu glu glu glu pro glu pro glu pro glu pro glu
841/281                                             871
GAG GAG GAA GCA GCA GAG GAG GAA GAC TAG AGT GAT AAC TGG TGG AAG GGC AAA GCC AAA
glu glu glu ala ala glu glu glu asp
901                                                 931
TAT ATC AAA TTC CTC AAG TGA TTA CTA GGA ATG AGA AGC TCA GGG AAG CAA AAG TTC AAT
961                                                 991
GGG AAC TGA CAG CTC TAA TGG CTC TAA CTA GGA CTC TAT ACT CTA AAA AGA CAC CAC TGT
1021                                                1051
AAG AGG ACC TAT TCC TCC ATG TTT CTC ATC TAA GTA AAT AAA TTC TTG TTT ACC TAG CAT
1081                                                1111
ATT AAA AAC AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA
```

Figure 3. Kyte-Doolittle hydrophobicity plot of mature PH-30 α. The Kyte-Doolittle hydrophobicity plot for mature PH-30 α was determined using the computer program DNA strider 1.1. The transmembrane domain and the putative fusion peptide are marked "tm" and "fp", respectively.

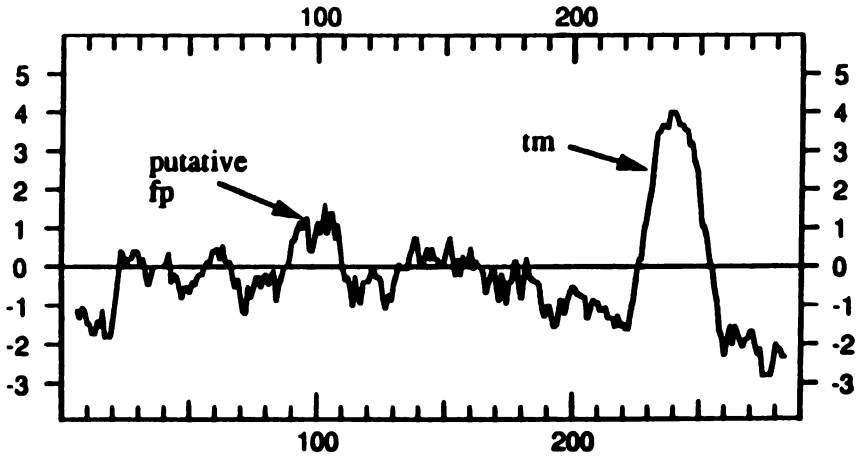

100
200
5
4
3
2
1
0
-1
-2
-3
putative
fp
tm
100
200

Fig 4. Nucleotide sequence of the lambda gt 11 cDNA clone encoding for mature PH-30 ß, and the deduced protein sequence. The nucleotide sequence of PH-30 was determined by direct sequencing of PCR products generated from a lambda gt 11 clone encoding for the mature PH-30 ß gene. The corresponding protein sequence was translated in the 3 letter amino acid code using the strider 1.1 computer program. A putative polyadenylation signal (AATAAA on the 3' end of the sequence) is underlined.

```
1/1                                                 31/11
CCC GTC TAC AGG TCA AAC CCG GTC TGC GGC AAT AAC AGG GTG GAA CAG GGT GAA GAC TGC
pro val tyr arg ser asn pro val cys gly asn asn arg val glu gln gly glu asp cys
61/21                                               91/31
GAC TGC GGA TCG CAG GAG GAA TGC CAA GAC ACC TGC TGT GAT GCT GCT ACC TGC AGG CTG
asp cys gly ser gln glu glu cys gln asp thr cys cys asp ala ala thr cys arg leu
121/41                                              151/51
AAA AGT ACT TCA CGA TGT GCT CAA GGG CCC TGT TGT AAC CAG TGT GAG TTC AAA ACT AAA
lys ser thr ser arg cys ala gln gly pro cys cys asn gln cys glu phe lys thr lys
181/61                                              211/71
GGA GAG GTA TGC CGA GAG TCC ACG GAT GAG TGT GAT CTC CCT GAG TAC TGC AAC GGC TCG
gly glu val cys arg glu ser thr asp glu cys asp leu pro glu tyr cys asn gly ser
241/81                                              271/91
TCT GGG GCT TGC CAA GAA GAC CTC TAT GTT ATT AAT GGG CAC AGA TGC GCA AAT GAA GAG
ser gly ala cys gln glu asp leu tyr val ile asn gly his arg cys ala asn glu glu
301/101                                             331/111
TGG ATC TGC ATG AAT GGG AGG TGT CTG TCT GGG AAG GCA CAA TGC CAA GAA ACA TTT GGT
trp ile cys met asn gly arg cys leu ser gly lys ala gln cys gln glu thr phe gly
361/121                                             391/131
ACA GAA ATG GAA ATG GGT TCA GTA GAC TGC TTT GAA CAA CTT AAT ACT AAG AAT GAC ATT
thr glu met glu met gly ser val asp cys phe glu gln leu asn thr lys asn asp ile
421/141                                             451/151
ACT GGA AAC TGT GGT ATC CTC AGC CCC GGG AAT TAC AAA GCA TGT GGA GCT AGC AAT TGG
thr gly asn cys gly ile leu ser pro gly asn tyr lys ala cys gly ala ser asn trp
481/161                                             511/171
AAG TGT GGA AAA TTA ATC TGT TCC TAT GAT AAG AGC GAA ATC CTG AGA AAC AAG GAA GGC
lys cys gly lys leu ile cys ser tyr asp lys ser glu ile leu arg asn lys glu gly
541/181                                             571/191
ATG ACC ATT TAT GCC AAC ATC AGC GGC CAT ATC TGT GTC AGC ATA GAA TAT CCT CCT GGT
met thr ile tyr ala asn ile ser gly his ile cys val ser ile glu tyr pro pro gly
601/201                                             631/211
CAC GCC AAG AGT GCA CTG ATG TGG GTA AGA GAT GGC ACC GTG TGT GGC CCG AGT GAG GTT
his ala lys ser ala leu met trp val arg asp gly thr val cys gly pro ser glu val
661/221                                             691/231
TGT AGG CAA CAA CAG TGT GTA TCC AGT TCG TAT TTG GGA TAT GAC TGC ACA CCA GCC ACT
cys arg gln gln gln cys val ser ser ser tyr leu gly tyr asp cys thr pro ala thr
721/241                                             751/251
TGC AGT GAT CAT GGT GTA TGC AAT AAC AAA AGG CAC TGT CAC TGT AAT CCC ACC TAT GTA
cys ser asp his gly val cys asn asn lys arg his cys his cys asn pro thr tyr val
781/261                                             811/271
CCT CCA AAC TGC GAG ACC CAA GAT TCG ACA AAG CCT GGA GGG AGT GTT GAC AGC GGT AAT
pro pro asn cys glu thr gln asp ser thr lys pro gly gly ser val asp ser gly asn
841/281                                             871/291
CTA CGA TAT GAA CCA ATC CCT GAA ACG TAT TTC GTT GAA GGT GCT TAC CAT ACC AAG TCT
leu arg tyr glu pro ile pro glu thr tyr phe val glu gly ala tyr his thr lys ser
901/301                                             931/311
AGA AAA TGG CCA TTT TTC TTG ATC ATT CCT TTT TTC GTT ATT TTC TCC GTA CTG GTT GCT
arg lys trp pro phe phe leu ile ile pro phe phe val ile phe ser val leu val ala
961/321                                             991/331
ACA GTG GTA AAA GTC TAT TAC CAA AAG AAA AAA TGG AAA ACT GAA GAT TAT GCA AAT GAT
thr val val lys val tyr tyr gln lys lys lys trp lys thr glu asp tyr ala asn asp
1021/341                                            1051/351
GAG AAC ATT GAA AGC GAG AGT GAA CCC AAA AGC TCC AAA GTC TCT TCC AAG TAG ACT GGC
glu asn ile glu ser glu ser glu pro lys ser ser lys val ser ser lys
1081                                                1111
TGG CAA GGA TTC TAT GCT GCC ACA GTG AGT GTC AAA TAG TTC AAG TCT TCC AGA ACA AGT
1141                                                1171
ATC TTT AGT GGA TAG AAA AAA GTG GAG AAG AAA AAA TAT GCA CTA CCT TAC TTC CTG GAA
```

1201 1231
GTC AAA CTG ATG TAT CGT GGT TCC AGT GCA CCA GAA ACT ATA GAC ATT TCA TGT ACA TGT
1261 1291
AAC ATA CAT ATA TGT CGT ATA TAT ATG ATT CTA TAA CAG ATA ATT TAT TTG TAA GGA AGG
1321 1351
CCT AAT TAT GAG TTT TAC ATT ACA TTT TTC TCA TTT TAA AAG TTA TTT CAC ACC GTG TTA
1381 1411
GCT AGA AGT CAC TAA TTC TGT TAG TAG GCA TGA TAT AGA AAA AGT TAT AAT AAA GGT AAT
1441
ACC ATG GGA A

Figure 5. Protein sequence of mature PH-30 ß. The protein sequence was translated in the 1 letter amino acid code from the open reading frame in the PH-30 ß cDNA clone using the strider 1.1 computer program. Peptide sequences that had been determined by automated Edman degradation.

VYRSNPVCGNNRVEQGEDCDCGSQEECQDTCCDAATCRLKSTSRCAQGPCCNQC
EFKTKGEVCRESTDECDLPEYCNGSSGACQEDLYVINGHRCANEEWICMNGRCL
SGKAQCQETFGTEMEMGSVDCFEQLNTKNDITGNCGILSPGNYKACGASNWKC
GKLICSYDKSEILRNKEGMTIYANISGHICVSIEYPPGHAKSALMWVRDGTVC
GPSEVCRQQQCVSSSYLGYDCTPATCSDHGVCNNKRHCHCNPTYVPPNCETQD
STKPGGSVDSGNLRYEPIPETYFVEGAYHTKSRKWFFLIIPFFVIFSVLVATV
VKVYYQKKKWKTEDYANDENIESESEPKSSKVSSK

Figure 6. Kyte-Doolittle hydrophobicity plot of mature PH-30 ß. The hydrophobicity plot of mature PH-30 ß was generated using the strider 1.1 computerprogram. It shows a transmembrane domain near the C terminus of the protein.

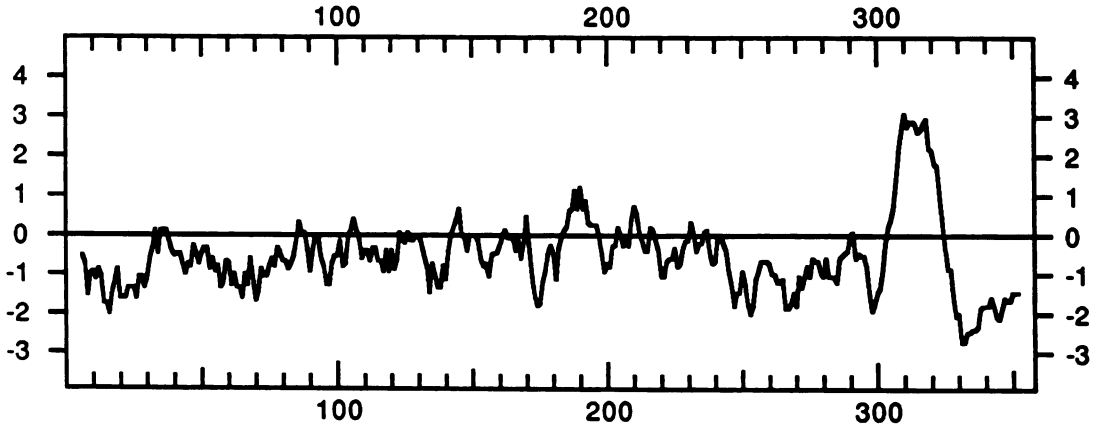
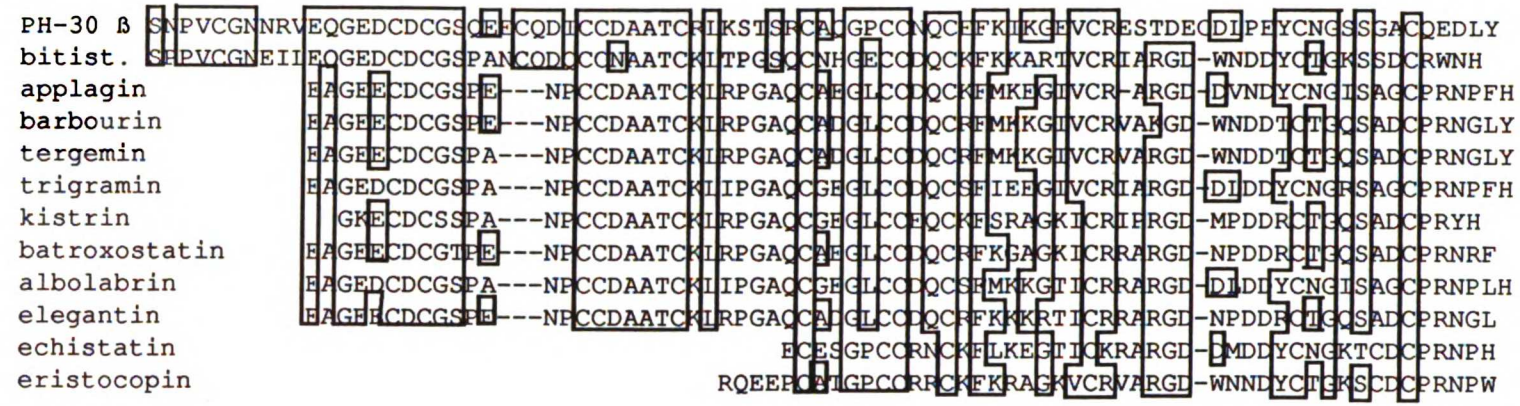

100
200
300
4
3
2
1
0
-1
-2
-3
100
200
300

Figure 7. Protein sequence alignment of PH-30 ß with snake venom platelet aggregation inhibitors and with laminin B2. The PH-30 ß protein sequence is aligned with snake venom platelet aggregation inhibitors to show regions of homology. The alignment with the protein bitistatin was generated by searching the genebank database with the PH-30 ß sequence. The other sequences were aligned visually and were taken from published protein sequences. The alignment with laminin B2 was found by searching the genebank database with the region of PH-30 (RESTDEC) that aligns with the RGD sequence of the snake venom peptides. Amino acids adjacent to the laminin B2 sequence RGSTDEC were aligned visually with PH-30 ß sequences and with the sequence of trigramin as an example for the snake venom peptides.


PH-30 ß
bitist.
applagin
barbourin
tergemin
trigramin
kistrin
batroxostatin
albolabrin
elegantin
echistatin
eristocopin

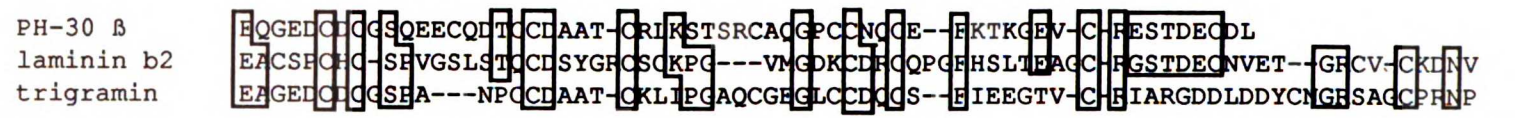
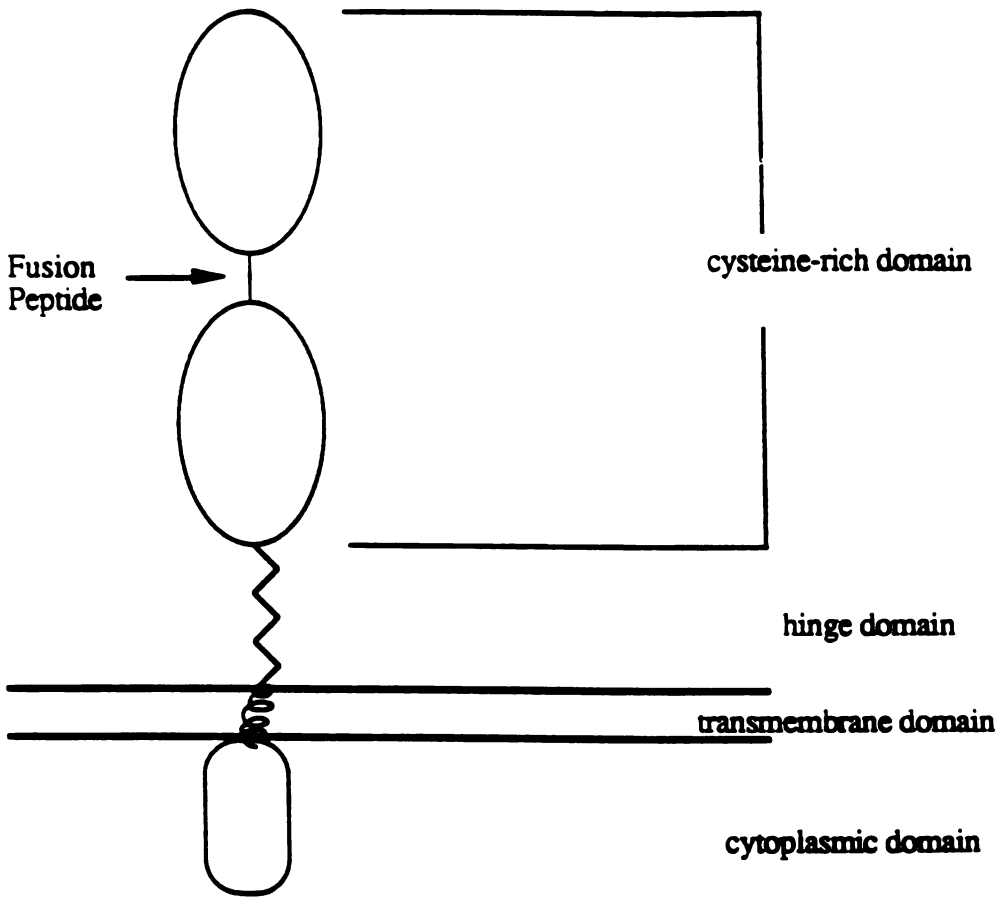

PH-30 ß
laminin b2
trigramin

Figure 8. Region of similarity between PH-30 α and the Rubella virus fusion protein E2. By searching the genebank database, the second hydrophobic region of PH-30 α was found to be similar to a region of the rubella virus fusion protein E2 that represents one half of a putative hydrophobic fusion peptide. Double dots indicate identical amino acids; single dots indicate conserved changes.

```
                                90
α                       GDSTKCGKLICTGISS
                        :  :.::.::: :.:.
Rubella Virus E2        GADTRCGRLIC-GLST
                          450         460
```

Figure 9. Predicted domain organization of PH-30 α. The domain organization of PH-30 α was predicted by Fernando Bazan (Fletteric lab). PH-30 α is predicted to have a cytoplasmic tail, a transmembrane domain, and two cysteine rich globular domains surrounding the putative fusion peptide.


Fusion
Peptide
cysteine-rich domain
hinge domain
transmembrane domain
cytoplasmic domain

CHAPTER 5

A Proteolytic Activity with a Possible Role in Sperm Maturation

ABSTRACT

We have previously shown that processing of PH-30 ß occurs in the epididymis concomitant with the acquisition of fertilization competence (Chapter 3). Here we present evidence that a proteolytic activity present on testicular sperm can process PH-30 ß in a fashion indistinguishable from that seen during epididymal maturation in vivo, however without epididymal contact. Other exogenously added proteases generate a clearly distinct processing pattern. This sperm associated activity can be inhibited by various protease inhibitors. Since it can mimic the proteolytic processing seen in vivo at the time sperm become fertilization competent we conclude that this proteolytic activity might play an important role in guinea pig sperm maturation.

INTRODUCTION

In transit through the epididymis sperm cells undergo a number of modifications that lead to sperm maturation. Sperm first become fertilization competent in the distal corpus epididymis, presumably through a combination of biochemical modifications of sperm proteins and the acquisition of motility by an unknown mechanism. The guinea pig sperm protein PH-30 is thought to play a role in sperm-egg fusion for the following reasons: Of two monoclonal antibodies that bind PH-30, one inhibits fusion with the plasma membrane of zona free eggs, whereas a second antibody does not [5]. PH-30 has features in common with viral fusion proteins. Cloning of PH-30 has revealed that it is a complex of two integral membrane glycoproteins. Processing of the ß subunit to its mature form occurs in the distal corpus epididymis, at the same time that sperm become fertilization competent. Fertilization competence might be acquired, at least in part by revealing the mature N-terminal domain of ß which is a putative ligand for an egg integrin (chapters 3 and 4). In analogy to several viral fusion proteins that require proteolysis for activation, this processing event might therefore play a role in activating PH-30. We have found a proteolytic activity present on testicular sperm that can process PH-30 in vitro, without contact with the epididymis, in a way indistinguishable from the maturation seen in vivo in the epididymis.

RESULTS

Testicular sperm were isolated on a Percoll gradient and washed extensively as described in Materials and Methods. The sperm cells were then disrupted by brief trituration in Ca++-Hepes buffer (without detergent) and incubated for 15 min on ice or at 37° C, or over night at 37° C in the absence of protease inhibitors (figs 1 B and D, lanes 1,2 and 3, respectively). After incubation, the cells were lysed, subjected to SDS PAGE, transferred to nitrocellulose and probed with a polyclonal antiserum against PH-30 (fig.1, A and B) or with a monoclonal antibody against PH-30 termed VC2 (fig.1 C and D). For comparison, figures 1 A and C depict western blots of guinea pig sperm cells at different stages of maturation (see below). The later samples were lysed in the presence of protease inhibitors (to prevent proteolysis), to demonstrate configurations of PH-30 encountered during in vivo sperm maturation. The PH-30 polyclonal antiserum binds to both the α and ß subunits of PH-30; and the monoclonal antibody VC2 recognizes PH-30 ß at all stages of sperm maturation. PH-30 ß was present in its precursor form, pre ß (MW 83 kD), on testicular sperm incubated in the presence of protease inhibitors (fig 1 A and C, lane 1) or on ice without protease inhibitors (fig 1 B and D, lane1). If testicular sperm were incubated in the absence of protease inhibitors at 37° C for 15' (fig 1 B and D, lane 2), pre ß (MW 85 kD) was processed to pre ß*, ß 1, and some ß 2 and ß 3 (with MW 75, 32 and 28 kD, respectively). After an overnight incubation at 37° C, pre ß had been converted to pre ß *, and to ß 1, ß 2 and ß 3. This processing pattern closely mimicked the pattern

encountered on sperm isolated from the distal corpus and proximal cauda epididymis (fig 1 A and C, lanes 2 and 3 respectively) where one finds pre ß* and ß 1, but no or very little pre-ß. During further epididymal maturation, the processing of ß proceeds and ß 3 appears in addition to ß 1 and ß 2. On distal cauda epididymal sperm (fig 1 A and C, lane 4), only ß 1, ß 2 and ß 3, but hardly any pre ß or pre ß * are visible. We have previously shown that PH-30 α is cleaved from pre α in the testis before the testicular stage of sperm maturation, and that it does not undergo any further processing. As expected, PH-30 α did not undergo visible processing either in vivo or in vitro under the conditions described here.

In contrast to testicular sperm, mature distal cauda epididymal sperm can be disrupted and incubated in the presence or absence of protease inhibitors without any visible effect on PH-30 ß as determined by western blot analysis (not shown).

The proteolytic activity described here could be completely inhibited by a cocktail of protease inhibitors (not shown). We therefore wished to determine more precisely which individual protease inhibitors were capable of preventing the activity. Testicular sperm were incubated as described above in the presence or absence of various protease inhibitors at 37° C for 4 hrs (see table 1). We found that the activity was strongly inhibited by soybean trypsin inhibitor, antipain, aprotinin, leupeptin, and benzamidine (see table for concentrations used). It was not noticeably inhibited by 1-10 phenanthroline, IAA, and EDTA, and it was weakly inhibited by TLCK, pepstatin, chymostatin and PMSF. We conclude that the

protease inhibitor spectrum points to the activity of a serine protease that does not require divalent cations or free sulfhydryl groups.

Next we wished to determine if the band pattern of PH-30 ß generated by incubation of testicular sperm is specific to the sperm protease or if it could be mimicked by other proteases as well. We have previously described processing of pre ß on intact testicular sperm by various added proteases such as trypsin, chymotrypsin, proteinase k and dispase. When digested to completion, these proteases generated a pattern similar, but not identical, to that seen on mature distal cauda epididymal sperm (see chapter 3). None of the proteases we tried on testicular sperm was able to precisely mimic the band pattern seen in vivo. Only the activity present on testicular sperm described here was capable of doing so. To further investigate the specificity of our assay in identifying a proteolytic activity, we incubated testicular sperm with the proteases mentioned above under conditions leading only to partial proteolysis. Specifically, 5 ug/ml of trypsin, chymotrypsin, proteinase k, and 20 ug/ml of dispase were added to intact testicular sperm at 37° C for 15 min. The resulting band pattern generated by partial digestion of pre ß with these four proteases on testicular sperm was clearly distinct from that seen in vivo, or that generated after incubation of disrupted testicular sperm in vitro. We conclude that the proteolytic activity present on testicular sperm is, at a minimum, at least very similar in specificity to that observed in vivo.

When testicular sperm were lysed in 1% NP40 and incubated at 37°C over night, a band pattern similar to that seen when testicular sperm were disrupted in the absence of detergent (as described above) was seen (not shown). This result implies that the processing pattern of PH-30 is not a result of constraints imposed by its membrane configuration, but is rather due to the specific conformation of the PH-30 protein. When the detergent extract was kept on ice, or incubated in the presence of protease inhibitors, no processing was seen. Even though the processing of PH-30 pre ß in the detergent lysate was very similar to that described above for disrupted testicular sperm, the ß3 band was not seen, even after long incubation periods at 37°C. It is unclear why ß3 is not obtained in a lysate. Perhaps this final cleavage step does rely on a certain configuration only present when PH-30 is in a membrane.

DISCUSSION and PERSPECTIVE

It is well established that sperm cells contain a number of proteases, many of which are stored in the acrosome. These are thought to be released during the acrosome reaction to allow penetration of the egg zona pellucida [10]. Metalloproteases released during the acrosome reaction have been shown to play a role in sperm-egg fusion [37, 39]. They have been proposed to process, and thus activate a putative sperm fusion protein [10]. With respect to PH-30, a putative sperm egg fusion protein, we have found that the acrosome reaction does not appear to lead to proteolysis or other changes, at least as assayed by western blot analysis. In this assay, processing of PH-30 in vivo appears complete by the time sperm undergo the acrosome reaction. (Metalloproteases could of course influence fusion by a number of means not visible in this assay, perhaps by inducing a conformational change of PH-30 or other proteins, or for example by removing sperm coat proteins that interfere with close membrane apposition.)

A proteolytic processing step of PH-30 that we propose could lead to its functional activation occurs instead at an earlier stage of sperm maturation in transit to the distal corpus epididymis (see also chapter 3). This kind of cleavage step is conceptually similar to proteolytic processing required to activate several viral fusion proteins. By assaying the proteolytic processing of PH-30 pre ß on western blots we have demonstrated that testicular sperm are a possible source for such a proteolytic activity. When highly washed testicular sperm are disrupted in the absence of protease inhibitors,

processing of PH-30 pre ß proceeds in a manner indistinguishable from that seen in vivo during sperm maturation in the epididymus. The processing does not occur on ice, indicating that the proteolytic activity itself could be present in a precursor (zymogen) form on testicular sperm [70]. We propose that in vivo this activity would be activated during epididymal maturation in the distal corpus epididymus, perhaps through contact of sperm with the epididymal tubules. This possibility requires hat the sperm protease that processes PH-30 pre ß be a cell surface protein (see below). A second possibility is that the epididymal tubules contain and secrete a proteolytic activity with the same specificity as the one we found on testicular sperm.

In the in vitro processing assay presented here the appropriate zymogen is presumably artificially activated through disruption of testicular sperm, and therefore does not require actual contact with the epididymis. A similar situation has been described in clamydomonas, where cell disruption is sufficient to release a protease that in turn activates a second protease involved in mating [71]. Cloning of the gene encoding the ß subunit of PH-30 suggests that the N-terminal region of ß functions as a ligand for an integrin present on the egg plasma membrane (chapter 4). If this is the case, then the proteolytic activity we have identified may play an important role in sperm maturation, perhaps by uncovering the PH-30 integrin (ligand) binding domain. The proteolytic activity described here could therefore be a target for a contraceptive

protease inhibitor. Conversely, a lack of this proteolytic activity might be responsible for certain cases of unexplained male infertility.

Future studies will aim at analyzing whether the proteolytic activity is present in isolated sperm membranes. We will test whether it behaves as an integral membrane protein, based on the criteria of TX114 fractionation and resistance to extraction with high salt or alkali. It will also be interesting to see if other sperm surface glycoproteins are affected by this protease. Specifically we plan to look at PH-20, a protein involved in binding of sperm to the zona pellucida, and at labeled (iodinated or biotinilated) surface proteins as a whole. We further surmise that the monoclonal antibodies PH-30 and PH-1, that do not bind to PH-30 on testicular sperm, should do so after proteolytic processing (see also chapter 3). Purification of the proteolytic activity might be accomplished by affinity chromatography using protease inhibitor columns, or by conventional biochemical means. The sequence of mature PH-30 ß has revealed an arginine residue immediately upstream of the N-terminus (see chapter 4), suggesting, as did our protease inhibitor studies, that a serine protease might be responsible for the processing of PH-30 ß *in vivo*. Purification and application of the activity might also be required for our attempts to reconstitute the putative membrane fusion activity *in vitro* following expression of the cloned PH-30 α and ß precursors in fibroblasts.

Table 1. Effect of various protease inhibitors on processing of PH-30 ß

Protease inhibitor	Concentration	Effect[a]
Soybean trypsin inhibitor	5 mg/ml	+++
Antipain	5 mg/ml	+++
Aprotenin	2 mg/ml	+++
Leupeptin	1 mg/ml	+++
Benzamidine	25 mg/ml	+++
Pepstatin	.5 mg/ml	+
PMSF	8.7 mg/ml	+
EDTA	.5 M	-
Iodoactamide	50 mg/ml	-
Phenanthroline	10 mg/ml	-

[a] The effect of the protease inhibitors was monitored by comparing the conversion of PH-30 pre ß to pre ß* and ß 1, ß 2 and ß 3 in a testicular sperm lysate on a western blot in the absence of protease inhibitors to the conversion in the presence of the inhibitors listed in this table. +++ indicates good inhibition of the proteolytic activity (very little conversion), + indicates some inhibition, and - indicates no inhibition.

Figure 1. Epididymal processing of PH-30 ß can be mimicked in vitro by a sperm associated protease. In vivo maturation of PH-30 is shown in panels A and C, and in vitro processing is shown in panels B and D. Panels A and B are stained with the PH-30 polyclonal antiserum, whereas panels C and D are stained with the monoclonal antibody VC2, which reacts with PH-30 ß only. Testicular sperm lysed on ice in the presence of protease inhibitors are shown in lane 1 of panels A and C; testicular sperm lysed in the absence of protease inhibitors on ice are shown in lane 1 of panels B and D. Lanes 2 and 3 in panels A and C show distal corpus epididymal sperm and proximal cauda epididymal sperm, respectively. Lane 4 in panels A and C depicts mature distal cauda epididymal sperm. Lanes 2 and 3 in panels B and D show testicular sperm incubated for 15 min or overnight at 37° C, respectively, in the absence of protease inhibitors.

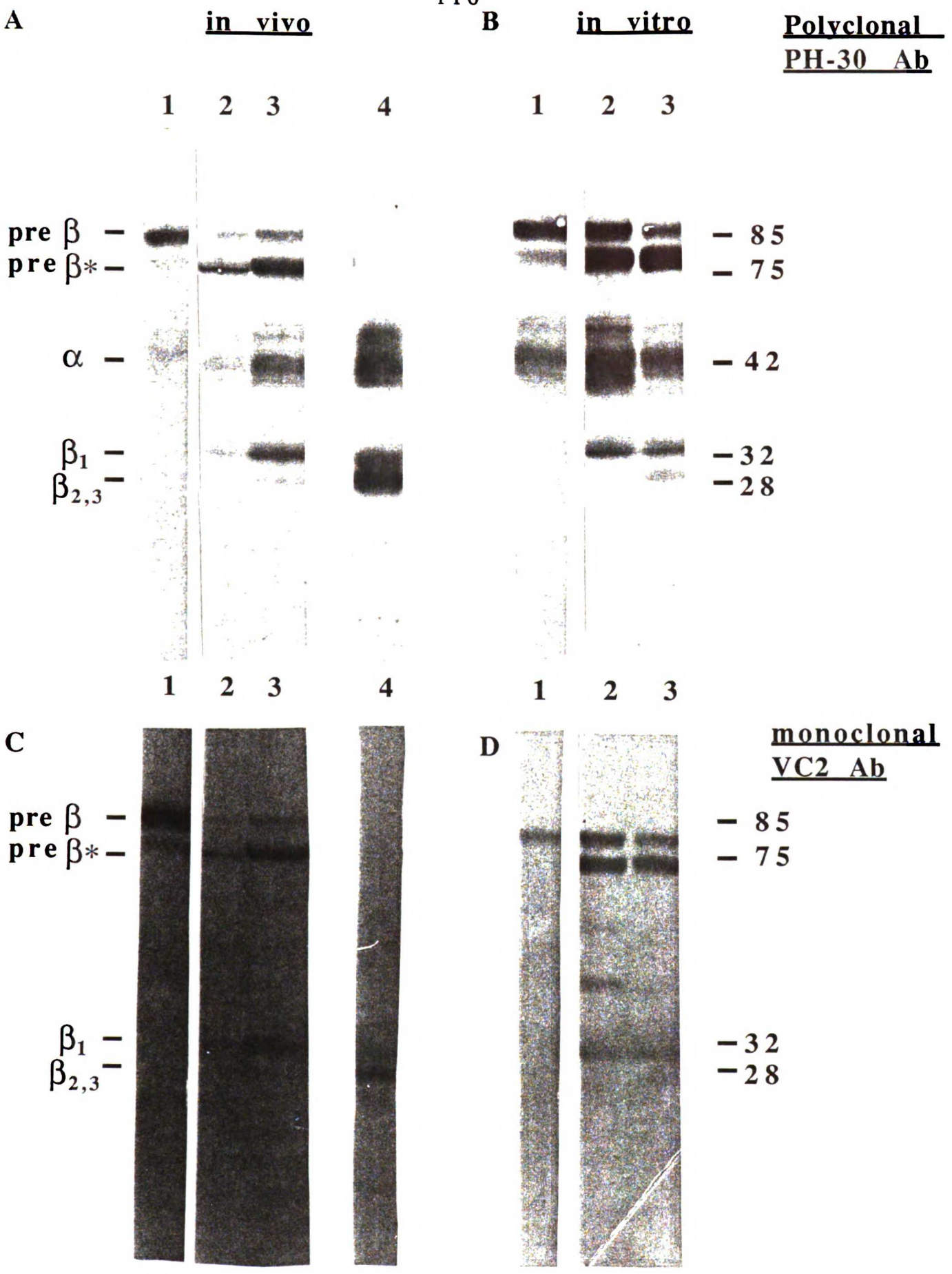
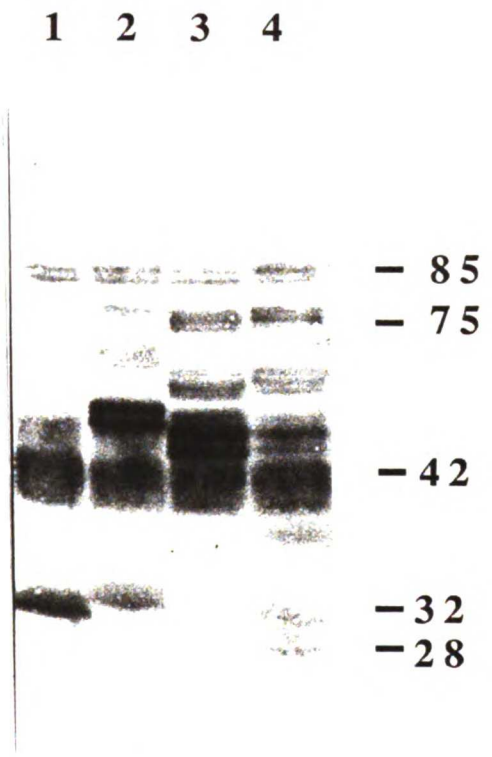

A
in vivo
B
in vitro
Polyclonal
PH-30 Ab
1 2 3 4
1 2 3
pre β
pre β*
α
β1
β2,3
85
75
42
32
28
1 2 3 4
1 2 3
C
D
monoclonal
VC2 Ab
pre β
pre β*
β1
β2,3
85
75
32
28

Figure 2. Added proteases create clearly distinct band patterns of PH-30 processing. Western blots of testicular sperm treated with 5 ug/ml each of trypsin (lane 1), chymotrypsin (lane 2), proteinase k (lane 3), or 20 ug/ml of dispase (lane 4) for 20 min at 37° C were probed with the polyclonal PH-30 antiserum.


1
2
3
4
85
75
42
32
28

CHAPTER 6

PH-30 Accessory Proteins

ABSTRACT and INTRODUCTION

The studies of PH-30 presented so far have focused on the α and ß subunits of PH-30 originally described by Primakoff and coworkers [5]. At least two other proteins, that we have termed "accessory proteins", copurify with PH-30 on a monoclonal antibody column. This chapter describes what has been learned about these proteins to date. On silver stained gels of nonreduced, affinity purified PH-30, these proteins appear as distinct bands of very high molecular weight in the stacking gel. When they are eluted from the stacking gel and run an SDS gel under reducing conditions, bands of MW 50 and 70 kD are seen. A polyclonal antibody raised against these proteins shows no crossreactivity with PH-30 α and ß, indicating that they are immunologically distinct. At this point it is unclear what role, if any, these accessory proteins might play in the fusion process. Further studies are outlined that might help shed light on this question.

RESULTS

When PH-30 was affinity purified, denatured, but not reduced , and run on preparative SDS gels (for subsequent protein sequencing) we always observed some very high molecular weight bands in the stacking gel (fig 1, lane 1) in addition to α, ß and the α/ß complex. When affinity purified protein was run on a gel under reducing conditions (fig 1, lane 2) two bands with apparent molecular masses of 50 and 70 kD respectively appeared in addition to PH-30 α and ß (apparent molecular masses 60 and 45 kD, respectively). These two newly identified proteins were termed PH-30 "accessory proteins" since they copurify with PH-30 on a monoclonal antibody column and are therefore most likely bound to PH-30 α and ß throughout the purification procedure. Alternatively the accessory proteins might bind directly to the mAb PH-30 by virtue of a shared epitope.

When a western blot analysis was performed with a polyclonal antibody raised against PH-30 protein (see below) on total sperm proteins, including those remaining in the stacking gel, bands with apparently identical molecular masses to those observed on silver stained gels were seen (fig 2, lane 3). The polyclonal PH-30 antiserum was raised against nondenatured and nonreduced PH-30 protein that was affinity purified on a monoclonal antibody column (ref). This antiserum was used in the biochemical characterization of PH-30 (chapter 1). We interpret the reactivity of this antiserum with the very high molecular weight accessory proteins as confirmation that the original protein preparations also contained these accessory

proteins, even though they were not described in the initial characterization (ref).

In order to learn more about these accessory proteins, a new polyclonal antibody was raised against material eluted from the stacking gel of purified PH-30 protein preparative gels. Since the original PH-30 antiserum reacts poorly with reduced PH-30 proteins, rabbits were immunized (at separate injection sites, but simultaneously) with both reduced and nonreduced accessory proteins. As shown in the western blots presented in fig 1, lanes 3-6, the resultant antiserum recognized both reduced, as well as nonreduced (SDS denatured) proteins. Lane 3 shows a western blot of reduced affinity purified PH-30 protein incubated with the accessory protein antiserum. The antiserum bound to bands of apparent molecular masses 50 and 70 kD respectively, corresponding to the bands seen on a silver stained gel of affinity purified and reduced PH-30 (fig 1, lane 2). In lane 4, an identical sample probed with the original PH-30 polyclonal antiserum showed only bands corresponding to PH-30 α and ß, with MW of 45 and 60 kD, respectively. When a reduced whole sperm extract was probed with either the PH-30 serum (lane 5) or the accessory protein serum (lane 6), the bands described above were seen in addition to a new band with apparent molecular mass of 30 kD. However, the 70 kD band was not as intense as on purified protein. The intensity of the α and ß bands recognized by the PH-30 antiserum on a whole sperm extract is also considerably weaker, most likely because there is relatively less PH-30 protein present in the whole extract lanes compared to

those with pure protein. These results imply that there is no immunological crossreactivity between PH-30 α or ß and the accessory proteins.

We next performed a western blot analysis on nonreduced sperm extracts (fig 2, lanes 1,2,4,5), or purified but nonreduced PH-30 protein (fig 2, lanes 3 and 6) with the PH-30 (lanes 1, 2 and 3) or the accessory protein antiserum (lanes 4, 5 and 6). The respective samples were either incubated at room temperature in 0.1% SDS (lanes 1 and 4), or boiled in 2% SDS for 5 min (lanes 2, 3, 5 and 6) prior to electrophoresis. As described in chapter 1, PH-30 α and ß run as a complex of MW 75 kD when not reduced and incubated at room temp in low SDS concentrations (fig 2 lane 1). When boiled in the presence of 2% SDS, the complex dissociates, and α and ß resolve separately with apparent molecular masses of 45 and 28 kD, respectively (fig 2 lane 2). When identical blots of sperm were incubated with the accessory protein anti-serum, identical patterns were seen whether the samples were incubated at room temperature in 0.1% SDS, or boiled in 2% SDS for 5 min before electrophoresis (fig 2, lanes 4 and 5, respectively). In both lanes a ladder of bands with increasing apparent molecular weights was seen, with distinct bands visible into the stacking gel. When a sample of purified nonreduced PH-30 protein was subjected to western blot analysis (fig 2, lane 6), the accessory protein antiserum reacted with bands corresponding to those seen (by silver staining, fig 1, lane 1) in the stacking gel of a sample of purified nonreduced PH-30 protein.

DISCUSSION AND PERSPECTIVE

We describe here the discovery of new proteins, termed "accessory proteins", that copurify with the α and ß subunits of PH-30 when it is affinity purified on a monoclonal antibody column. These proteins are either bound to PH-30 throughout the column purification step, or they might be bound by the mAb PH-30 directly because they share an epitope with PH-30 ß. The accessory proteins appear to be highly oligomeric, since they are found as very high molecular weight bands on SDS polyacrylamide gels. They are recognized by the original PH-30 polyclonal antiserum on western blots when they are nonreduced. This antiserum does not react with the accessory proteins when they are reduced. We therefore raised a new polyclonal antibody that binds to both reduced and nonreduced accessory proteins. Using this antiserum, the accessory proteins were shown to be immunologically distinct from the PH-30 α and ß subunits. The new antiserum recognizes the high molecular weight accessory proteins, but not α or ß, on western blots of purified PH-30 protein. However, on a western blot of whole sperm extracts more bands are visible; these seem to form a ladder, perhaps corresponding to increasing stages of oligomerization. These results imply that only the most highly oligomeric multimers of the accessory proteins copurify with PH-30 α and ß, even though more forms exist in a sperm extract. At present we have no good explanation for this finding. One might speculate that there is something unique and perhaps also of functional significance about the higher molecular weight forms of the accessory proteins that

allows them to bind to PH-30. We believe that the ladder of bands recognized by this antiserum on nonreduced sperm extracts are not nonspecific background bands for two reasons. First, they are not seen by the preimmune serum to the accessory protein antiserum (not shown). Second, all of the accessory protein bands seem to consolidate into two bands of apparent molecular masses 50 and 70 kD, respectively, when the sperm extract is reduced. This does not rule out the possibility that nonspecifically reacting bands might change their conformation when reduced. In this context it is interesting to note that the PH-30 polyclonal antiserum only reacts on western blots with the high molecular weight accessory proteins, similar to those that copurify on the mAb PH-30 column; the lower molecular weight forms of the accessory proteins that are recognized by the new accessory protein antiserum are not recognized by the PH-30 polyclonal antiserum. This result might imply that the conformation of the accessory proteins bound to PH-30 is different from those that are not. An alternative explanation for the copurification of the "accessory proteins" with PH-30 might be the presence of a shared epitope. Given that affinity purified polyclonal antibodies and the new polyclonal antibody against reduced PH-30 ß both do not bind to the accessory proteins, we believe that this explanation is unlikely.

In the future it will be interesting to see if the accessory proteins are bound only to PH-30, or if they also bind to other proteins on the sperm surface. Preliminary evidence suggests that at least the very high molecular weight forms are largely absent from a sperm extract

that has been immunodepleted with the mAB PH-30 (not shown), indicating that at least these forms are not bound to other proteins. However, further experiments are needed to confirm this finding. It is quite possible that the accessory proteins, or at least the lower MW forms, are bound to other proteins on the sperm surface. This question could be addressed by immunoprecipitation experiments under varying conditions, with PH-30 monoclonal (mAb PH-30, PH-1 and VC2) or polyclonal antibodies, and with the accessory protein serum. Sucrose gradient analysis and crosslinking experiments would provide further evidence and characterization of a complex between α, ß and the accessory proteins. If the accessory protein antiserum functions in immunofluorescence experiments, we will determine whether the accessory proteins, like PH-30, are restricted to the posterior head of the surface of mature epididymal sperm. We further wish to establish if the accessory proteins are membrane proteins, if they are glycosylated, when they first appear in spermatogenesis, and if they are also made as precursors that undergo processing during sperm maturation. If these results indicate that the association of the accessory proteins with PH-30 is specific, we must consider the possibility that these proteins play a role in sperm egg fusion. It might then be of interest to clone the gene(s) for these proteins for further analysis. Given that PH-30 is a ligand for integrins (chapter 4), it is possible that the sequence of the accessory proteins will reveal some homology to other members of the integrin superfamily.

Fig 1. Accessory proteins copurify with PH-30 on a monoclonal antibody affinity column. Silver stained gel of nonreduced and reduced PH-30 protein (lanes 1 and 2). Western blots of reduced sperm proteins probed with the antiserum against PH-30 and the PH-30 accessory proteins (lanes 3 - 6). Lane 1 shows a silver stained gel of PH-30 protein that was affinity purified on a monoclonal antibody column, boiled in 2% SDS for 5 min. and analyzed under nonreducing conditions by SDS-PAGE. Under these conditions the α and ß subunits of PH-30, the α/ß complex, and the accessory proteins in the stacking gel are visible. In lane 2 a silver stained gel of reduced purified PH-30 protein is shown. In addition to PH-30 α and ß, two major bands of MW 70 and 50 kD are visible. Lanes 3 and 4 depict western blots of purified reduced PH-30 protein, and lanes 5 and 6 show western blots of whole distal cauda epididymis sperm extracts. Lanes 3 and 6 were incubated with the accessory protein antiserum; lanes 4 and 5 were incubated with the PH-30 polyclonal serum.

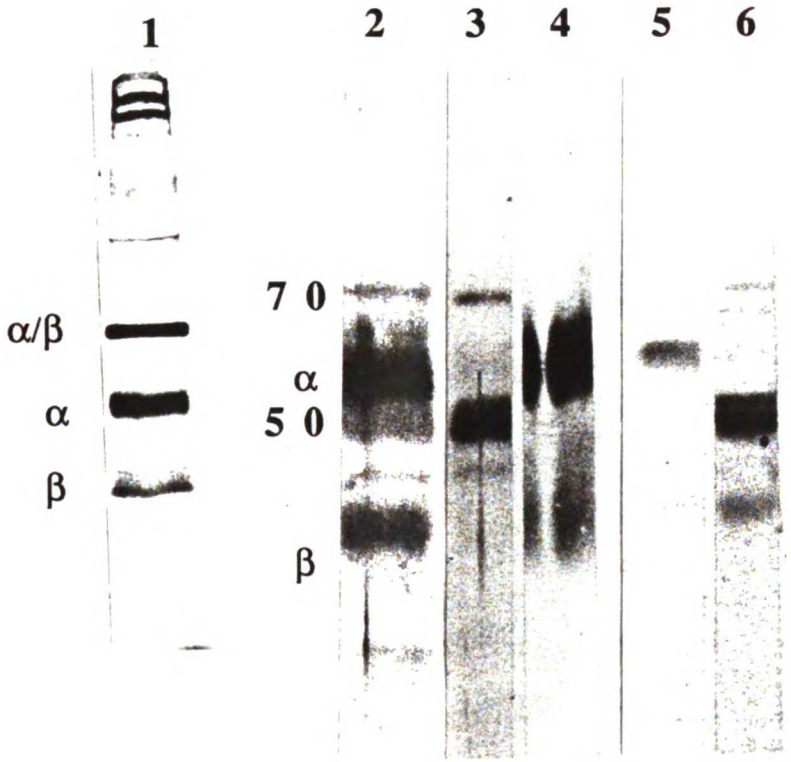

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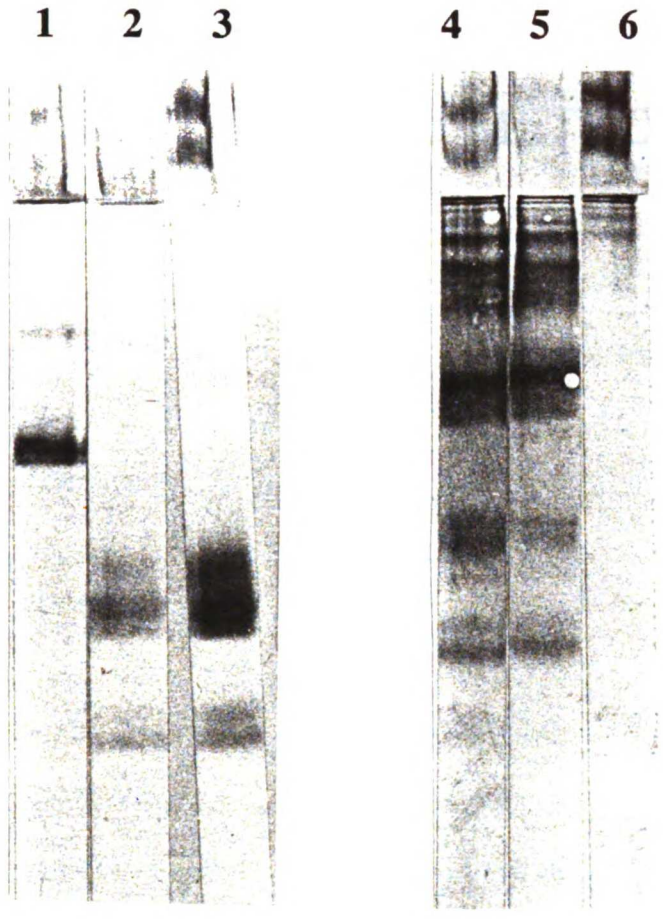
Fig 2. Western blots of nonreduced PH-30 protein or sperm extracts probed with the polyclonal PH-30 anti-serum and the polyclonal accessory protein anti-serum. Distal cauda epididymal sperm extracts were incubated in 0.1 % SDS at room temperature (lanes 1 and 4), or boiled in 2 % SDS prior to electrophoresis and transfer to nitrocellulose (lanes 2 and 5). Purified PH-30 protein was boiled in 2 % SDS prior to electrophoresis and transfer (lanes 3 and 6). Lanes 1-3 were incubated with the PH-30 polyclonal anti-serum; and lanes 4-6 were incubated with the accessory protein anti-serum.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MATERIALS and METHODS

Antibodies

The primary antibodies used in this study were monoclonal antibodies, mAb PH-30 and mAb PH-1 [5], and a rabbit polyclonal antiserum raised against nondenatured PH-30 affinity-purified on a mAb PH-30-Sepharose column [5]. IgG from the polyclonal antiserum was purified on a column of Protein-A Sepharose (Sigma Chemical Company, St. Louis, MO). For immunoblot analyses, primary antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse IgG (Promega Corp., Madison WI). For immunofluorescence analyses, primary antibodies were detected with rhodamine-conjugated goat anti-rabbit or goat anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN).

Preparation of gametes

Sperm from male Hartley guinea pigs (retired breeders) were collected from three regions of the epididymis: the distal corpus epididymis, the proximal cauda epididymis and the distal cauda epididymis. These regions correspond to region II, region IV and region VI/VII, respectively, of the guinea pig epididymis as described by Hoffer [72]. Testicular cells and testicular sperm were collected from the testis and separated on a 52% isotonic Percoll (Sigma Chemical Co.) gradient in Mg^{++}-Hepes buffer by centrifugation for 10 min at 27,000 x g, 10°C. Isolated gametes

were then washed twice in Mg^{++}-Hepes buffer (0.14 M NaCl, 4mM KCl, 4 mM Hepes, pH 7.4, 10 mM glucose, 2 mM $MgCl_2$ (8).

Preparation of gel samples, electrophoresis, and immunoblot analysis

Cells were lysed in a nonionic detergent containing cell lysis buffer (1% NP40, 50 mM Tris, 2 mM EDTA, pH 7.4). Protease inhibitors (1mM PMSF, 1 μg/ml pepstatin A, 2 μg/ml leupeptin, 4 μg/ml aprotinin, 10 μg/ml antipain, 50 μg/ml benzamidine, 10 μg/ml soybean trypsin inhibitor, and 100 μg/ml iodoacetamide) were added to the cell lysis buffer just prior to use. All protease inhibitors were from Sigma Chemical Co. Typically ~ 4 x 10^6 cells were lysed in 100 μl cell lysis buffer and 25 μl of the extract was analyzed per gel lane. Samples were either boiled in sample buffer containing 2% SDS or incubated at RT in sample buffer containing 0.5% SDS as indicated in the text. Samples were not reduced prior to electrophoresis. Electrophoresis was conducted according to Laemmli [73], with minor modifications, on 10% resolving gels with 5% stacking gels. Prestained molecular weight standards (Sigma Chemical Co.) were: α2-macroglobulin (180,000 D), β-galactosidase (116,000 D), fructose-6-phosphokinase (84,000 D), pyruvate kinase (58,000 D), fumerase (48,000 D), lactic dehydrogenase (36,000 D), and triose phosphate isomerase (26,600 D). For immunoblot analyses (16), proteins were transferred to nitrocellulose (Schleicher and Schuell, Inc., Keene, NH), and the nitrocellulose sheets were blocked with 5% dry milk

reconstituted in distilled water. Incubation with primary antibodies, alkaline phosphatase-conjugated secondary antibodies (Promega Corp.), and color development with 5-bromo, 4-chloro indolyl phosphate and nitroblue tetrazolium (BCIP/NBT; Promega Corp.) were performed following the Promega Corp. protocols and applications guide. Apparent molecular weights were calculated with reference to the prestained molecular weight markers.

Immunodepletion of material reactive with mAb PH-30.

An extract of mature epididymal sperm was immunodepleted of mAb PH-30 reactive material by passage over a column containing a large excess of mAb PH-30 coupled to cyanogen bromide-activated Sepharose CL-4B (14). To verify the immunodepletion, the column flow through was passed a second time over the column. A sample of the material eluted from the second column was analyzed by SDS-PAGE. Silver staining [74] of the gel revealed that all of the mAb PH-30 reactive material had been removed during the first passage over the column.

Digestion with endoglycosidase F.

Distal cauda epididymal sperm were lysed in cell lysis buffer and adjusted to 10 mM sodium acetate (pH 5.5), 0.1% SDS and 1.5 mM PMSF. Samples (10 µl) containing 30 µg of sperm protein and 0.5 units of endoglycosidase F (Boehringer Mannheim) were incubated overnight at 37°C. Mock digested samples were prepared and incubated as above in the absence of added enzyme.

Binding to Concanavalin-A Sepharose.

Concanavalin-A (Con-A) Sepharose CL-4B beads (100 µl; Sigma Chemical Co.) or control Sepharose CL-4B beads (100 µl; Sigma Chemical Co.) were washed twice in cell lysis buffer and incubated on ice for 1 h with an extract of $5x10^6$ distal cauda epididymal sperm lysed in 100 ul cell lysis buffer. The beads were centrifuged for 10 min at 12,000 x g in a microfuge. Unbound material was saved and adjusted to 2% SDS in sample buffer. The beads were then washed 4 times with 500 ul cell lysis buffer and the bound material removed by boiling in sample buffer (2% SDS). After removing the beads by centrifugation (10 min at 12,000 x g), the samples were analyzed by SDS-PAGE.

Micro-affinity purification of antibodies

Micro-affinity purification of antibodies was conducted essentially as described by Gluck and Caldwell (7). The proteins in a boiled extract of epididymal sperm were separated by preparative SDS-PAGE and transferred to nitrocellulose. Nitrocellulose sheets were blocked as described above and incubated with a 1:20 dilution of PH-30 rabbit polyclonal antiserum for 1 hr at RT. A narrow strip was cut from both sides of the blot, incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega Corp.), and developed with BCIP/NBT (Promega Corp.) as described above. A strip corresponding to the desired band was then cut from the nitrocellulose sheet and placed into a 15 ml Falcon tube. Bound antibodies were eluted with elution buffer (5 mM glycine, 500 mM NaCl, 0.1% BSA, 0.5% Tween 20, pH 2.5) for 1 minute, and immediately neutralized with a predetermined amount of saturated $NaHPO_4$. The strip was then washed with PBS, 0.05 % Tween 20, followed by two more elution and wash cycles.

Proteolytic treatment of testicular sperm

Testicular sperm were washed and resuspended at a concentration of 10^7 sperm/ml in Mg^{++}-Hepes buffer. For immunoblot analysis, testicular sperm were incubated for 2 h at 37°C with 20 ug/ml proteinase K (Sigma Chemical Co.), chymotrypsin (Boehringer Mannheim), or L-1-Tosylamide-2-phenylethyl chloromethyl ketone

(TPCK)-treated trypsin (Sigma Chemical Co.), or with 100 ug/ml dispase (Boehringer Mannheim). At the end of the incubation, the protease inhibitor cocktail described above was added to the cells and the cells were then lysed in cell lysis buffer, and prepared for SDS-PAGE as described above. Approximately 1 x 10^6 testicular sperm were analyzed per lane. Testicular sperm to be analyzed by immunofluorescence microscopy were incubated on ice for 1 hr with 5-fold higher concentrations of proteases: 100 ug/ml proteinase K, chymotrypsin or trypsin, or 500 ug/ml dispase. Digestion with 5-fold higher protease concentrations on ice generated immunoblot patterns identical to those seen when testicular sperm were treated with the the lower concentrations of proteases at 37° C (not shown).

Incubation of disrupted testicular sperm in the presence or absence of protease inhibitors.

Testicular sperm were prepared as described above, washed three times in Ca-Hepes buffer, pelleted in a microfuge, and then either frozen in liquid nitrogen for later use or disrupted immediately by rapid trituration (5 passages through a 200 ul pipettip) in a volume of 100 ul/ 10^6 cells. The disrupted cells were then either incubated on ice, or at 37°C for different
lengths of time. The incubation at 37°C was performed in the absence of protease inhibitors, or, to asses the effect of individual protease inhibitors, with either soy bean trypsin inhibitor (5 mg/ml), antipain (5 mg/ml), aprotenin (2 mg/ml), leupeptin (1 mg/ml), benzamidine (25 mg/ml), pepstatin (.5 mg/ml), PMSF (8.7 mg/ml), iodoactamide

(50 mg/ml), 1-10 phenanthroline (10 mg/ml) or EDTA (.5 M). Following incubation, the cells were pelleted in a microfuge a second time, lysed and subjected to electrophoresis on SDS gels as described above. The effect of the individual protease inhibitors was assessed by comparing the relative amount of conversion of PH-30 pre ß in the absence of protease inhibitors to that seen in the presence of the inhibitors listed above.

Indirect immunofluorescence microscopy

Indirect immunofluorescence staining was performed on live sperm as described previously. Primary antibodies were detected with rhodamine-conjugated goat anti-mouse or goat anti-rabbit IgG (Boehringer Mannheim).

Generation of PH-30 protein sequence information

Reagents

High purity electrophoresis grade reagents were used in all buffers and solutions for protein purification. SDS (Sigma Chemical company, St. Louis, MO) was recrystalized as described below to remove any contaminants. This is thought to be crucial according to some protocols, although others allow use of highest purity SDS commercially available from Bio Rad or USB. I found the use of

recrystalized SDS to be essential compared to regular Sigma SDS, and used it in all buffers and solutions used for protein purification.

Recrystalized SDS

100g SDS were added to 450 ml ethanol, stirred and heated to 55°C. 50 - 75 ml hot water were added until all of the SDS dissolved. Then 10 g activated charcoal were added, (if possible in a 42°C oven to prevent SDS crystalization as it cools). The filtrate was chilled for 24 hrs at 4°C, then another day at -20°C. The SDS was then collected in a glass filter funnel with a white porous filter support without filter. It was washed with 800 ml ethanol (-20°C). Then the same procedure was repeated without charcoal treatment. The SDS was then dried under vacuum overnight at room temperature.

Preparative PAGE

Preparative electrophoresis for protein purification was performed essentially as described above, except that the running buffer was made up containing 0.1% recrystalized SDS, and 0.1 mM sodium thioglycolate (a radical scavenger) was added. The concentrated protein sample was mixed with an equal volume of 2x sample loading buffer made with recrystalized SDS. For subsequent elution of α and ß,the sample was incubated at 95°C for 10 min without reducing reagents, allowed to cool and then loaded onto the gel. When nonreduced, α and ß separated better from the accessory proteins that copurify with PH-30 on the monoclonal antibody

column(see also chapter 6). For amino terminal sequencing on immobilon membranes the sample was boiled, reduced, and alkylated prior to SDS PAGE.

Electroelution of proteins from a polyacrylamide gel slice

The purified PH-30 protein was subjected to PAGE and visualized by staining in 0.5% Coomassie blue in 15 % Acetic acid, 15 % methanol for 15 min, followed by a 20 min. rinse in H_2O. Bands corresponding to PH-30 α, ß and the accessory proteins were then excised. The band of interest was placed in a chamber of an "Elutrap" electroelution apparatus (Schleicher and Schuell, assembled according to the manufacturers instructions) containing elution buffer (0.5 M NaH_3HCO_3; 0.1 % recrystalized SDS). The gel slice was soaked in elution buffer for 30 min before the electroelution was started. The elution was performed at 80 V for 2 days. Buffer was recirculated to avoid pH changes using a peristaltic pump at a rate of 2 - 4 drops/ second. Eluted samples were removed about every 12 hrs and stored in an eppendorf tube at - 20°C.

Concentration and SDS removal

The eluted protein samples were concentrated in a speedvac (lyophilizer) to remove volatile NaH_3HCO_3. The samples were then resuspended in 100 ul H_2O, and precipitated with 900 ul acetone , 1 mM HCl (stored at - 20°C) for at least 3 hrs to overnight at - 20°C to remove SDS (SDS interferes with CNBr and proteolytic cleavage

reactions). The precipitated protein was then pelleted for 30 min in a microfuge at 4°C, washed twice with 200 ul ice cold acetone, and allowed to air dry. The protein pellet was subsequently subjected to either CNBr, V8 or tryptic digest, as described below. Each cleavage step was monitored by running 1 % of the total sample on a gel which was then silver stained as described above.

Cleavage with CNBr

For CNBr cleavage (cleaves at met residues), the protein pellet was resuspended in 200 ul 70% formic acid containing 1 mg/ml CNBr (200 ug cleaves 100 ug of most proteins). The sample was incubated in the dark under argon for 24 hrs. It was then diluted 5 fold in H_2O, and lyophilized in a speed vac concentrator to dryness. The process was repeated after adding 500 ul ddH_2O to remove the remaining traces of formic acid. The cleaved protein was then either run directly on an SDS PAGE for subsequent blotting onto immobilon membranes, or digested a second time with trypsin before separation on reverse phase HPLC.

For PAGE, the protein was resuspended in 1 x sample buffer, reduced with 25 mM DTT for 10 min at 95°C, alkylated with 50 mM IAA for 30 min at room temperature and then electrophoresed on a 12 % acrylamide gel, followed by transfer to immobilon. The transferred bands were stained with Coomassie blue, excised and subjected to automated Edman degadation.

Trypsin digest

For trypsin digestion, the precipitated purified protein was either resuspended in 200 ul 5 mM NH_3HCO_3, 0.1% SDS, or in 50 ul 8 M urea, 0.4 M NH_3HCO_3, which was heated to 50°C for 15 min and diluted with 150 ul H_2O (final concentration: 2 M urea, 0.1M NH_3HCO_3). 2 - 5 ug trypsin were then added (a 1/10 - 1/25 ratio of enzyme/protein), and incubated for 24 h at 37° C. An amino acid analysis was performed on a large protein preparation indicating that about 2.2 nM protein had been obtained from 64 giunea pigs, corresponding to 70 -85 ug of protein. Usually, 20 guinea pigs were sacrificed for each individual attempt to obtain protein sequence, corresponding to about 25 ug or 700 pM of protein.
The peptides resulting from trypsinization were then separated on a reverse phase HPLC and then subjected to protein sequencing by automated Edman degradation..

V8 digestion

For V8 digestion the samples were resuspended in 100 ul 5 mM NH_3HCO_3, 0.1% SDS as described above, and incubated at 37°C for 24 h with 2 ug V8 (1/10 - 1/20 ratio enzyme protein). An equal volume of 2 x SLB was added, and the sample was boiled, reduced and alkylated, as described above, and subjected to SDS PAGE followed by transfer to immobilon membranes.

Design and synthesis of oligonucleotide primers for PCR and sequencing

Degenerate oligonucleotide primers were designed based on the protein sequence obtained from purified PH-30 α and ß. If possible, a G or C was placed at the 3' end of the primer. The primers were either ordered from the UCSF Biomolecular Resource Center, or synthesized by members of the Julius lab on an Applied Biosystems Oligonucleotide synthesiser. The oligonucleotide primers were eluted from the resin by incubation in ammoniumhydroxide at 55°C for at least 5 hrs according to manufacturers instructions. The ammoniumhydroxide was then removed in a speedvac concentrator, and the oligonucleotides were resuspended in H_2O for use as primers.

Polymerase Chain Reaction

Reactions requiring the polymerase chain reaction to identify the genes for PH-30 α and ß were performed as recommended by Perkin Elmer/Cetus. Briefly, a standard reaction was made up of 5 ul DNA template, 2 ul 10x PCR buffer (Perkin Elmer/Cetus), 2 ul of each primer at a concentration of 50 pM and 2 ul of a 2 uM stock solution of dNTP's. Immediately prior to temperature cycling, 1/8 ul of taq polymerase was added to the reaction, which was overlayed with a drop of mineral oil to prevent evaporation. The reaction was then subjected to 40 cycles of 1 min incubations at 92°C for denaturation, 40 - 50°C for annealing (depending on the GC content of the primers used), and 74°C for extension, respectively.

Gel purification and direct sequencing of DNA generated by PCR

DNA generated in a PCR was run on a 5% acrylamide gel in 1x TBE (Maniatis laboratory manual), visualized under UV illumination after staining with EtBr, excised and eluted over night in H_2O. The following day the DNA was EtOH precipitated and subsequently resuspended in 6.25 ul H_2O. 1.25 ul sequencing primer at a concentration of 5 pM and 2 ul of 5x sequencing buffer (all sequencing reagents were obtained from USB) were added. The solution was then heated for 2 min at 95°C, and then immediately frozen in a dry ice/EtOH bath, and allowed to thaw on ice. The subsequent steps were performed following the protocol provided by USB with the sequenase sequencing kit.

Screening a lambda gt 11 library with ^{32}P labeled DNA probes

Growth of lambda gt 11 phage for the purpose of screening a library, transfer of phage colonies to nitrocellulose filters, denaturation and hybridization of ^{32}P labeled probes were performed as described in the molecular cloning manual by Maniatis. The 230 basepair fragment of PH-30 ß was labeled with ^{32}P using a random primed labeling kit (BRL) according to the manufacturers suggestions.

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