



IDENTIFICATION OF LINEAR SURFACE EPITOPES ON THE GUINEA PIG SPERM MEMBRANE PROTEIN PH-20

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Summary

The sperm plasma membrane protein PH-20 has previously been shown to be an effective immunogen for protection against fertilization in guinea pigs. To identify immunodominant regions on gpPH-20 that may be related to this contraceptive effect, we used several high-titer immune sera obtained from animals rendered infertile by gpPH-20 injections to screen a set of overlapping peptides that cover the entire 494-residue sequence. Multiple clusters of peptide sequences exhibited specific reactivity. Some of these sequences were then constructed as octameric synthetic peptides and tested for immunogenicity in female guinea pigs. Our results indicated two regions (res. 94-119 and res. 424-444) to be highly immunogenic and both are surface accessible when native gpPH-20 is in solution or anchored on sperm surface. Both anti-peptide antibodies are specific for gpPH-20 and one of them inhibited hyaluronidase activity partially. These monospecific antibodies should be useful probes for further molecular definition of gpPH-20 structure-function relationships.

Key Words: PH-20, sperm protein, hyaluronidase activity, epitope mapping

Mammalian fertilization is a multi-step process involving the capacitation of sperm, its passage through the cumulus cells, the binding of sperm to the zona pellucida, and fusion with the egg plasma membrane. Specific surface molecules on the sperm migrate from one domain to another during the progression of these steps and the associated functional significance is under investigation in many laboratories. Several of these proteins have been identified and characterized (1, 2, 3), including PH-20. This molecule was originally identified by monoclonal antibodies directed against the posterior head region of guinea pig spermatozoa, and in later studies has been localized on sperm plasma membrane as well as acrosomal membrane (4, 5). Considerable *in vitro* evidences suggested that PH-20 functions during fertilization as a fusion receptor in the binding to zona pellucida of the egg (5, 6, 7). In addition, the protein catalyses the hydrolysis of hyaluronan in the extracellular matrix (6, 8, 9) and aids in the penetration of the cumulus cell layer. In *in vivo* experiments, immunization of male and female guinea pigs with purified gpPH-20 has been shown to result in infertility (10, 11). The contraceptive effect in male animals is long-lasting and at least partially reversible; whereas the effect in female animals is long-lasting and reversible. These studies clearly established that gpPH-20 plays an important

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role during fertilization in guinea pigs and suggested that similar functional significance may be found for its analogs in other species.

Homologous PH-20 proteins are widely distributed in many mammalian species and cDNA sequences of the guinea pig, mouse, macaque and human form of PH-20 have been determined (12,13,14). The deduced amino acid sequences of the various subtypes exhibit a high degree of homology, with structural features such as distribution of cysteine residues and potential N-linked glycosylation sites particularly conserved. The amino-terminal region of PH-20 encompassing about 300 residues is significantly homologous to hyaluronidase from a variety of sources including bovine and ovine sperm, bee and hornet venom and human plasma (14, 15, 16, 17, 18). To date, all forms of native and recombinant PH-20 studied have been demonstrated to possess intrinsic hyaluronidase activity (6, 9, 14, 15, 19, 20). The full-length protein is membrane-anchored through covalent linkage to glycosyl phosphatidylinositol moiety and could be released as a soluble hyaluronidase by PI-PLC treatment. (19, 21, 22). In addition, a proteolytic event during the acrosome reaction cleaves the molecule between arg-311 and ser-312, resulting in two polypeptide fragments linked through disulfide bonds (23). The exposed carboxy-terminal region has been hypothesized to be the binding domain for zona pellucida (6).

Given the essential functional role of PH-20 in fertilization, there has been substantial interest in its potential application as a contraceptive vaccine for humans. For the development of a viable vaccine candidate based on PH-20, it is important to define the detailed molecular structure of this protein. However, there is currently very limited information on the functional domain structure of PH-20, and essentially no information available on the antigenic determinants. A recent mutagenesis study identified five positions essential for the hyaluronidase activity (24), but deletion studies have not yielded a clear definition of the minimal structure of the catalytic domain (9, 24). Other studies generated different monoclonal and polyclonal antibodies to PH-20 which inhibit the sperm-egg binding and hyaluronidase activity to varying degrees (5, 6, 21). However, efforts to map the epitopes for various monoclonal antibodies have not been successful (6), and these epitopes of interest are most likely to be discontinuous in the primary sequence. An important trend emerging from these studies is that reactivity of the monoclonal reagents is species-specific, despite the high degree of sequence homology among the PH-20 proteins from different species. For example, the anti-gpPH-20 monoclonal antibodies do not cross-react with PH-20 from other species (13). The polyclonal sera directed against one type of PH-20 react only weakly with other types (13). It would thus be important to elucidate the epitopes uniquely defined by these antibodies as the regions are likely to be related to the species specificity of mammalian gamete interaction.

To explore the developmental feasibility of a recombinant peptide-based vaccine for contraception, we initiated a series of studies to define the immunogenic regions of PH-20. Peptide libraries were screened to define linear epitopes, while random phage libraries were screened to define mimotopes. In the present study, we mapped continuous epitopes of gpPH-20 by Pepscan analysis using several high-titer sera obtained from previous studies (10) in which gpPH-20 was affinity-purified from sperms and the immunized animals were rendered infertile. These regions were then constructed as octameric synthetic peptides and tested for immunogenicity in female guinea pigs. We defined two antigenic regions on gpPH-20 and characterized the reactivity of the corresponding antipeptide antibodies with native gpPH-20.

Methods

Materials: Hyaluronic acid (from human umbilical cord), bovine testes hyaluronidase (type IV), cetylpyridinium chloride, complete Freund's adjuvant and incomplete Freund's adjuvant were purchased from Sigma Chemical Co. (St. Louis, MO). Secondary reagents in ELISAs and Westerns were from Zymed Laboratories (South San Francisco, CA) or Sigma. Protein-A resins were purchased from Bioprocessing Inc. (Princeton, NJ). Nested peptide sets were custom synthesized on polyethylene pins by Chiron Mimotopes Pty Ltd. (Victoria, Australia). Octameric peptides were custom synthesized by Research Genetics (Huntsville, AL). Immune sera against native guinea pig PH-20 (gpPH-20), recombinant cynomolgus macaque PH-20 (cPH-20), recombinant mouse PH-20 (mPH-20), and ascites fluids containing various monoclonal antibodies were obtained from P. Primakoff's lab (U. Connecticut, Farmington, CT). Male and female Hartley guinea pigs were obtained from Charles River Laboratories (Boston, MA). Different PH-20 subtypes were purified from guinea pig epididymus, and baculovirus-infected Sf9 cell culture expressing cPH-20 or mPH-20 through a three-step process using medium-pressure liquid chromatography to be described elsewhere (C. P. Chan, manuscript in preparation).

Pepscan ELISA: Decapeptides spanning the published sequence of gpPH-20 (from residue 1 to 494) (12) were synthesized on polyethylene pins in 12x8 block format according to Geysen *et al.* (25). All peptides were N-acetylated and represent sequences that overlap by 7 residues (i.e. sequentially offset by 3 residues). For each peptide block, two control peptides were included for the synthesis and assays. ELISAs were carried out by incubating the pins with 200 μ l per well of reagent in 96-well microtiter plates on a slow rocking platform. Three washes of phosphate-buffered saline (PBS) were included between steps. To reduce non-specific binding, the pins were blocked with 2% bovine serum albumin (BSA)/0.1% Tween-20/PBS for 1 hour at 37°C. Incubation with the various primary antibody samples were carried out for 18 hours at 4°C. Immune serum samples (from female guinea pigs immunized with affinity-purified gpPH-20 obtained in a previous experiment (10)) and non-immune controls were assayed at 1:1000. Ascites containing monoclonal antibodies were assayed at 1:100. Horseradish peroxidase-conjugated anti-guinea pig IgG polyclonal antibodies from rabbit were then used at 1:4000 as detecting reagent. After incubation for 1 hour at 37°C, the pins were placed in the substrate solution (2,2'-azino-di(3-ethylbenzthiazoline sulfonic acid in 0.1 M sodium citrate/0.01% w/v hydrogen peroxide) for 30 min at room temperature. Aliquots of 140 μ l were then transferred to a clean microtiter plate and absorbance at 405 nm was recorded using a SpectroMax 250 multiwell plate reader (Molecular Devices, CA). To remove bound reagents from the pins after each assay, the polyethylene blocks were sonicated in 1% SDS/0.1% 2-mercaptoethanol/PBS for 10 min at 55°C, rinsed in 60°C distilled water, immersed in methanol, and finally air dried.

Peptide Immunization: Peptides with selected sequences (see Table I) were synthesized on solid supports with branched lysine cores by 9-fluorenylmethoxycarbonyl chemistry according to Tam (26). Resin-cleaved peptides were lyophilized, dissolved in PBS and sonicated for at least 1 hr before use. Amino acid compositions were verified by analysis on a Beckman 6500 system. For immunization experiments, 20 female guinea pigs (35-40 days of age) were divided into five treatment groups and one control group, with 3-4 animals per group. All animals were maintained and used in accordance with the Institutional Animal Care and Use Committee Guidelines. Each animal received 0.1 mg of peptide (or PBS for the controls) emulsified in Freund's complete adjuvant at multiple subcutaneous sites, and subsequently boosted twice with 0.1 mg of peptide emulsified in Freund's incomplete adjuvant at 2-week intervals in a similar

manner. Serum samples were collected immediately prior to each immunization and assayed against 0.2 mg/ml of immobilized peptides (on Immulon II plates, Dynatech Lab) by standard ELISA procedures. Horseradish peroxidase-conjugated anti-guinea pig IgG polyclonal antibodies from rabbit was used at 1:4000 for detection, and absorbance at 405 nm was recorded as described above. Values are considered significant when absorbance signals are two-fold above that of reagent controls (averaged=0.1). Titers are reported as the reciprocal of the highest serum dilution that resulted in significant signals.

Western analysis: SDS polyacrylamide gel electrophoresis was carried out on 12% Tris-glycine gels and the samples transferred to polyvinylidene difluoride (PVDF) membranes using a 4-buffer system (27). Blots were cut into strips and separately probed with different serum samples diluted to 1:100. Alkaline phosphatase-conjugated anti-guinea pig IgG goat serum was used at 1:2000, and 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium was used as enzyme substrate for detection.

Hyaluronidase Assay: Hyaluronidase activity was assayed in a microplate format as described previously (28) with some modifications. For each well assay, the substrate consisted of 100 μ l of 0.8 mg/ml hyaluronic acid in 0.3 M sodium phosphate, pH 5.7, and samples or standards were added in 10 μ l aliquots. After a 1-hr incubation at 37 °C, 100 μ l of 10% (w/v) 1-hexadecylpyridinium chloride were added, and the mixture incubated at room temperature for 5 min. Optical density at 595 nm was recorded using a SpectroMax 250 plate reader. A standard curve of 0.4 to 12 units was constructed using bovine testes hyaluronidase (750-1500 units/mg) and enzymatic activities in unknowns were calculated using curve-fitting software provided with the plate reader. For enzyme inhibition experiments, different PH-20 preparations were first incubated with various serum samples for 1-hr at 37 °C before testing of hyaluronidase activity as described above. Sera collected from normal guinea pigs and rabbits were used as controls. Relative activity is expressed as a percent of that of the controls. For immunoprecipitation experiments, serum samples at varying concentrations were first incubated with purified gpPH-20 overnight at 4°C. An equal volume of a 50% slurry of Protein-A resins were then added and the mixture shaken for 2 hr at room temperature. After pelleting by centrifugation, the supernatants were taken and assayed for residual hyaluronidase activity as described above. Sera collected from the control group in the immunization experiment were used as controls (100%). Relative activity is expressed as a percent of that of the controls.

Indirect Immunofluorescence Staining: Fresh cauda epididymal sperms were collected from male guinea pigs, washed with PBS, and then incubated at room temperature for 60 min with either control sera or immune sera at 1:100. After pelleting at 1000 x g, the suspension was incubated with fluorescein isothiocyanate (FITC)-conjugated anti-guinea pig rabbit sera at room temperature for 60 min. The sperms were then washed, resuspended in PBS, and spotted on microscope slides. The samples were viewed with a Zeiss Axioplan epifluorescence microscope fitted with FITC-specific filter sets, and the images were captured using a camera with Kodak Ektapress 1600 plus film. For each sample, phase contrast photographs were also taken, with 30-50 sperm cells examined per sample.

Results

Identification of dominant linear epitopes on gpPH-20 protein. Sera from three female guinea pigs rendered infertile by multiple immunizations of affinity-purified gpPH-20 (10) were used to screen peptide blocks covering the entire sequence of gpPH-20. ELISA results from the three serum samples were averaged and presented in Fig. 1A; overlay of the three data sets gave very similar profiles (individual data sets not shown). Several clusters of peptide pins gave ELISA signals between 1 to 2 units while most pins gave signals lower than 0.5, indicating specificity against the hyperimmune sera. To further control for non-specific reactivity, sera derived from guinea pigs that were not immunized were tested against the same peptide set. As shown in Fig. 1B, most peptide pins gave ELISA signals below 0.5 except for a few isolated ones and the cluster representing residues 355-382. In addition, reactivity of two monoclonal antibodies specific for gpPH-20 were compared to a non-relevant anti-peptide antibody (anti-KT3) using the same peptide blocks. Both mAb-20 & mAb-21 have been shown to immunoprecipitate gpPH-20 but do not bind in Western blotting experiments (5, 10). These results indicated the epitope sequence is conformation sensitive and likely to be discontinuous. Nevertheless, report on other proteins (29) demonstrated the utility of hexapeptide sets in the mapping of epitopes consisting of linear sequences discontinuous in the primary sequence. We proceeded with testing of the monoclonal reagents in the present study; however, no peptide pins with significant reactivity against these two antibodies were found (data not shown).

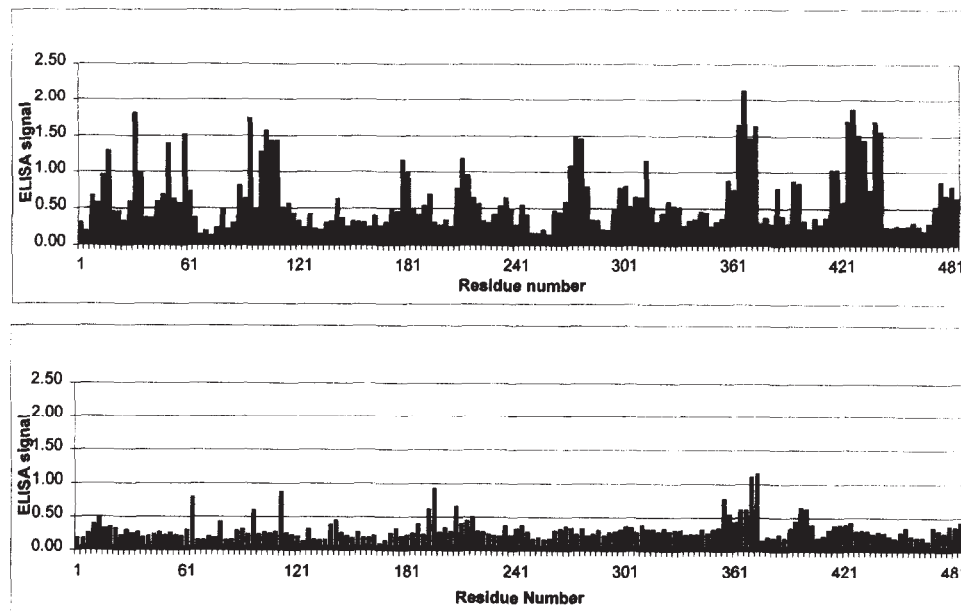


Fig. 1.

Pepscan screening of hyperimmune sera against overlapping peptides spanning the full-length sequence of gpPH-20. Linear 10-residue peptides were synthesized on blocks of polyethylene pins as described in **Methods**. The pins were probed for immunoreactivity with: (A, top) sera from infertile female guinea pigs, averaged values of 3 samples, (B, bottom) sera from non-immune guinea pigs, average of 2 samples. Absorbance units are plotted for each peptide. Residue number refers to the number of the first residue in each peptide with respect to the sequence of gpPH-20.

In selecting peptide sequences for the immunization experiment, we focused on continuous sequences represented by clusters of at least three pins that gave significant ELISA signals. In addition, sequences that are highly hydrophobic as predicted by secondary structure calculation routines (residues 298-315 and residues 475-502, using the Wisconsin/GCG package per ref. 30), and sequences that showed reactivity not specific to the hyperimmune sera (residues 355-382) were excluded. Five regions of sequences, 19 to 26 residues in length, representing residues 1-23, 46-65, 94-119, 268-286 and 424-444 (sequences shown in Table I) were thus selected for testing of immunogenicity in subsequent experiments.

TABLE I

Amino Acid Sequences of Octameric Peptides Used to Produce Anti-gpPH-20 Antibodies.

Peptide #	Residue # on gpPH-20	Amino acid sequence
1	1-23	DKRAPPLIPNVPLLWVWNAPTEF
2	46-65	NITGQSITLYYVDRLGYYPY
3	94-119	DILFYMP TDSVGLAVIDWEEWRPTWT
4	268-286	YIRLVFTDQTTTFLLEDDL
5	424-444	TANNICDAVLNFPSLDDDDDE

Production of anti-peptide antibodies. Throughout the immunization experiment, serum antibody responses were monitored by ELISA against the immunizing peptides. Table II showed a comparison of the level of serum responses to the various gpPH-20 peptides at the end of the study.

TABLE II

ELISA reactivity of serum samples against gpPH-20 peptides.

Peptide #	<i>Serum IgG response (absorbance units)</i>	
	1:100	1:1000
1	0.939 ± 0.203	0.231 ± 0.065
2	0.236 ± 0.048	0.173 ± 0.045
3	2.449 ± 0.420	1.799 ± 0.403
4	0.231 ± 0.042	0.126 ± 0.015
5	2.530 ± 0.080	2.060 ± 0.119

Serum samples were assayed at 1:100 and 1:1000 dilution against 0.2 mg/ml immunizing peptides by ELISA as described in **Methods**. Results are shown for samples collected after three injections (one prime and two boosts) and assayed twice. Each treatment group consisted of 3-4 animals and averaged absorbance values ± SEM are given.

Samples collected from control (PBS/adjuvant only) animals were also assayed against the 5 peptides, and showed no significant antibody responses (data not shown). There was no IgG response generated against peptides 2 and 4, and only a limited serum IgG response for peptide 1. This peptide sequence encompassed the N-terminal segment of gpPH-20 up to the first conserved cysteine residue and is highly homologous among the PH-20s from guinea pig, mouse,

cynomologous monkey and human. The low ELISA signal for the first four pins in the Pepsan analysis (Fig. 1) suggest that the N-terminal part of this sequence may not be exposed in the native form of gpPH-20. In contrast, serum IgG responses to peptides 3 and 5 were highly significant. Peptide 3 encompassed a conserved region in the different forms of PH-20 and contained several hydrophobic residues. Peptide 5 consisted of a C-terminal component where gpPH-20 is most divergent from the other forms. End-point titers for the immunizing peptides were subsequently determined by two-fold serial dilutions and titer values of 16,000 were found for both groups. In comparison, hyperimmune sera directed against native gpPH-20 yielded titers of 64,000 for peptide 3 and 32,000 for peptide 5. These results indicated that both regions are highly immunogenic.

Effects of anti-peptide antibodies on gpPH-20 protein. We proceeded to characterize the reactivity of the anti-peptide-3 and anti-peptide-5 antibody preparations against different forms of PH-20 protein. In this study, partially purified native gpPH-20 derived from guinea pig epididymus and recombinant mPH-20 and cPH-20 generated from baculovirus/Sf9 cell cultures were used. Fig. 2 showed the binding of various serum samples to the different PH-20 proteins electrophoresed under non-reducing conditions in a Western analysis.

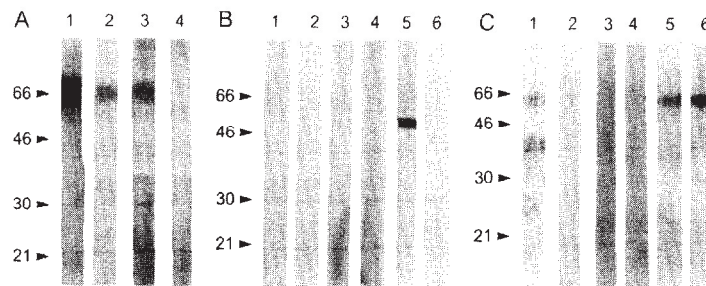


Fig. 2.

Immunoblotting analysis of various PH-20 proteins by anti-gpPH-20 peptide antibodies. Purified preparations of the different forms of PH-20 were electrophoresed without reducing agents on separate 12% Tris-glycine polyacrylamide gels and blotted onto to PVDF membranes as described in **Methods**. Membrane strips were independently probed with different serum samples at 1:100 dilution as follows: (**panel A**) gpPH-20 probed with: (lane 1) guinea pig anti-gpPH-20, (lane 2) anti-peptide 3, (lane 3) anti-peptide 5, (lane 4) control guinea pig serum; (**panel B**) mPH-20 probed with: (lane 1) guinea pig anti-gpPH-20, (lane 2) anti-peptide 3, (lane 3) anti-peptide 5, (lane 4) control guinea pig serum; (lane 5) rabbit anti-mPH-20, (lane 6) rabbit anti-cPH-20 serum; (**panel C**) cPH-20 probed with: (lane 1) guinea pig anti-gpPH-20, (lane 2) anti-peptide 3, (lane 3) anti-peptide 5, (lane 4) control guinea pig serum; (lane 5) rabbit anti-mPH-20, (lane 6) rabbit anti-cPH-20 serum

For gpPH-20 (Fig. 2A), a single diffused band of apparent MW ~66 kDa was seen for the PVDF strip probed with the hyperimmune sera used in the Pepsan analysis. This apparent molecular

size and broad electrophoretic mobility is similar to the findings of others (4, 23) and may be a consequence of the carbohydrate moieties attached to native gpPH-20 as well as some degradation over the course of purification. The two anti-peptide antibodies are clearly reactive with gpPH-20. The apparently lower signal intensities may be a combined result of the fact that these are mono-specific antibodies which only probe for a single epitope, and lower concentrations of reactive antibodies in the sera. For the mPH-20 preparation (Fig. 2B), a single band of ~52kDa was seen when the blot was probed with a rabbit serum directed against recombinant mPH-20, and indicated adequate amounts of reactive material on the blot. However, none of the other sera tested at identical dilutions (1:100) showed any reactivity. For the cPH-20 preparation (Fig. 2C), a specific rabbit serum gave a diffused positive signal at ~60kD. While this indicated some degradation of the preparation, comparison of serum cross-reactivity is still possible. The rabbit serum directed against mPH-20 showed cross-reactivity with multiple bands at slightly lower intensity, while the anti-gpPH-20 guinea pig sera showed weak but above background reactivity. Again, the control serum and the two anti-peptide sera showed no reactivity. Taken together, these results indicated that the two anti-peptide sera can bind full-length gpPH-20 and are relatively specific for the guinea pig form of PH-20 protein.

We then assessed the effects of the anti-peptide antibody preparations on the enzymatic activities of the different forms of PH-20 hyaluronidases. For each PH-20 preparations, a specific immune serum was included in the test set as a positive control for enzyme inhibition. In all cases, a control serum from the same host type was included to correct for non-specific effects in the assays.

TABLE III

Inhibition of various PH-20 hyaluronidases by specific hyperimmune sera.

Antiserum added	<i>Hyaluronidase activity (% control)</i>		
	gpPH-20	mPH-20	cPH-20
<i>guinea pig sera</i>			
control	100.0	100.0	100.0
anti-gpPH-20	8.5	106.6	67.0
anti-peptide 3	82.4	96.1	84.1
anti-peptide 5	90.3	93.7	89.6
<i>rabbit sera</i>			
control	100.0	100.0	100.0
anti-gpPH-20	7.6	104.8	91.5
anti-mPH-20	106.6	21.4	99.4
anti-cPH-20	105.7	102.3	19.5

Different preparations of purified PH-20 containing 5-10 units of hyaluronidase activity were preincubated with the various sera (1:50 final dilution) for 1-hr at 37 C before addition into the hyaluronidase assay mixture as described in **Methods**. For each host type (guinea pig or rabbit), a control serum sample was included. Data presented are expressed as percent of the control values. Experiments were repeated three times with duplicate incubations (SEM range=0.8-4.2).

As shown in Table III, the sera directed against gpPH-20 inhibited gpPH-20 enzyme activity.

The anti-peptide 5 antibodies had no effects while the anti-peptide 3 antibodies consistently showed a marginal (18%) inhibition. The results with the mPH-20 preparation paralleled the results of the Western analysis. Only the anti-mPH-20 rabbit sera inhibited mPH-20 enzymatic activity. For the cPH-20 preparation, it was interesting to note that the anti-gpPH-20 serum significantly inhibited the enzymatic activity (33%) while the Western cross-reactivity was minimal. On the other hand, anti-mPH-20 serum does not affect hyaluronidase activity despite significant cross-reactivity in Western analysis (Fig. 2). The anti-peptide 3 antibodies showed a weak (16%) inhibition of cPH-20 but not mPH-20 hyaluronidase. The anti-peptide 5 antibodies showed no inhibition of either the mouse or cynomolgus macaque form of PH-20 hyaluronidase.

To further characterize the interactions of the mono-specific antibodies with native gpPH-20 in solution phase and on sperm membrane (as opposed to the denatured, immobilized state in Western blots), we tested the ability of the sera to immunoprecipitate gpPH-20 and to stain live guinea pig sperms. Table IV showed the effects of the antibodies against peptide 3 and 5 on the removal of hyaluronidase activity from a partially purified preparation of gpPH-20. About 60-80% of enzyme activity was removed from the supernatants by the anti-peptide antibodies while the hyperimmune sera directed against gpPH-20 resulted in complete removal. As the partial degradation of the gpPH-20 preparation most likely contribute to this lack of complete precipitation, no further titration was attempted beyond a dilution of 1:50.

TABLE IV

Immunoprecipitation of hyaluronidase activity by anti-gpPH-20 peptide antibodies.

Antibody added	<i>Residual hyaluronidase activity (%)</i>
Control	100.0
Anti-gpPH-20	2.2
Anti-peptide 3	20.6
Anti-peptide 5	36.1

Purified gpPH-20 containing ~10 units of hyaluronidase activity was independently incubated (at 1:50 dilution) with control, anti-gpPH-20, anti-peptide 3 or anti-peptide 5 polyclonal antibodies derived from guinea pigs, and hyaluronidase activity in the supernatants were assayed after immunoprecipitation as described in Methods. Data presented are expressed as percent of the control values. Experiments were repeated twice with duplicate incubations (SEM range=0.8-4.2).

Fig. 3 showed indirect immunofluorescence staining of intact guinea pig sperms using antibodies directed against peptide 3 and peptide 5. Serum sample used in the Pepsan analysis (directed against gpPH-20 protein) was included as a positive control. Comparison with phase contrast images (not shown) indicated over 90% staining of sperms examined for all immune samples. In all three cases, we observed two patterns of sperm staining: intense signal on the posterior head (Fig. 3, A, B & C, top row) and intense signal on the whole head (Fig. 3, A, B & C, bottom row), with a ratio of 1:2. A control serum sample in comparison showed no significant staining; only a faint outline of the sperms could be discerned (Fig. 3D, phase contrast images not shown). We concluded from these results that the epitopes recognized by the mono-specific antibodies are surface-accessible in the native form of gpPH-20 in solution, and these regions remain surface accessible when gpPH-20 is anchored in the plasma membrane.

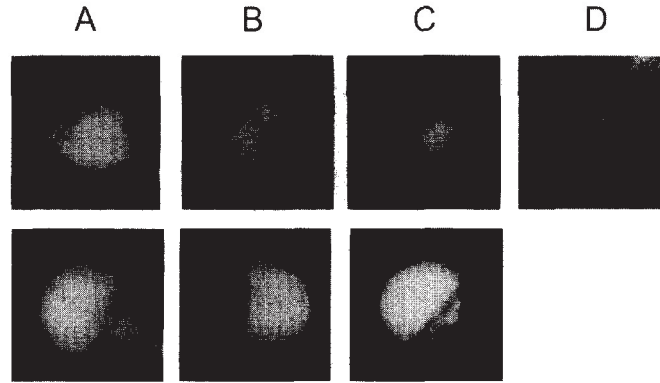


Fig. 3.

Immunofluorescence staining of guinea pig sperm preparation by anti-gpPH-20 peptide antibodies. Live sperms were stained with: (A) anti-gpPH-20 serum, (B) anti-peptide 3 serum, (C) anti-peptide 5 serum and (D) control guinea pig serum as described in **Methods**. Two types of labeling patterns were observed: intense staining on posterior head (top row) and staining on whole sperm (bottom row). Final magnification is $\sim 1000\times$.

Discussion

The present study was undertaken to define regions of contiguous sequences on gpPH-20 that are immunodominant which may be related to protective immunity against fertilization. The particular sera used in the Pepsican analysis were derived from the original study where the contraceptive effects of gpPH-20 were demonstrated in female guinea pigs (10). All the peptides exhibiting significant reactivity in Fig. 1 are thus potentially important in the generation of immune responses against fertilization. We have constructed a set of peptides that were sequentially offset by 3 residues to quickly define linear epitopes on the gpPH-20 protein with medium resolution. When some of these sequences were tested as linear peptides for immunogenicity, several peptides tested negative. This result does not necessarily indicate that these sequences are entirely non-antigenic. Future experiments in fine mapping using shorter peptides with single residue offsets may be employed to precisely define the epitopes before evaluation of *in vivo* effects. For the two regions of sequences that we have clearly identified as immunogenic and surface accessible, similar fine mapping experiments could be carried out to further define the minimal sequences involved and the optimal presentation of the epitopes. In particular, the marginal inhibition of anti-peptide 3 antibodies on gpPH-20 and cPH-20 hyaluronidase activities indicated that the peptide 3 sequence and other residues in proximity may be involved in enzymatic activity. Future experiments using shorter nested sets of peptides will be necessary to address the possibility of further minimizing the sequence for definition of the functional domains.

Mammalian gamete fusion involves multiple steps and is in most cases species specific. The dual functionality of PH-20 as both a digestive enzyme and an adhesion molecule, and the finding that multiple isoforms of the protein exist in the sperms of a given mammalian species (6, 19, 21, 22) suggested that PH-20 may function at multiple stages of the fertilization process. For delineation of functions, three murine monoclonal antibodies for gpPH-20 (4, 5) and various rabbit

polyclonal sera for gpPH-20, mPH-20 and cPH-20 (4, 6, 9, 19) have been generated in previous studies. The cross-reactivity of the various reagents to the different forms of PH-20 in protein binding and enzyme inhibition studies shown here and in part by other (7, 12, 21) provide an emerging definition of the functional domains of PH-20. However, the use of hyperimmune sera with multiple binding specificities could not provide definitive characterization of the molecule. Monoclonal antibodies are mono-specific, but attempts to map their epitopes (see our results and discussion in ref. 6) have not been successful. Our approach is to systematically derive mono-specific anti-peptide antibodies to complement existing reagents and define distinct sequences of potential functional interest. Here, we have identified two regions on gpPH-20 as highly immunogenic and developed mono-specific antibodies as probes for further studies. On the basis of the results from Western analysis, immunoprecipitation and live sperm binding studies, the anti-peptide antibodies for these two regions can bind to both the native and denatured forms of gpPH-20. The first region corresponds to residues 94-119 and lies within the N-terminal domain which is presumed to confer hyaluronidase activity (9, 14). This region is highly conserved among the different forms of PH-20, with gpPH-20 more homologous to cPH-20 (21 positions identical) than mPH-20 (18 positions identical) over the stretch of 26 residues. In the present study, the anti-peptide antibodies for this region weakly inhibited the gpPH-20 and cPH-20 hyaluronidase activities. A paper (24) published after the conclusion of our study indicated that mutation of residues Asp-111 and Glu-113 in the human PH-20 sequence substantially diminish hyaluronidase activity. Both residues are covered by peptide-3 in our study. These results confirmed the effectiveness of our approach, and strongly indicate that the mono-specific anti-peptide 3 antibodies is an important reagent for the dissection of PH-20 functions. The second region corresponds to residues 424-444 and lies within the C-terminal region which is associated with adhesion activity (6). It is also a region on PH-20 that is most divergent among different species. We demonstrated here that the anti-peptide 5 antibodies specifically react with gpPH-20 and not mPH-20 nor cPH-20. The identification of this specific region in gpPH-20 suggests that corresponding sequences in PH-20s from other species dictate the species specificity of mammalian gamete interactions.

In summary, we have defined two regions of sequences on gpPH-20 that are immunodominant and surface accessible in the native state. Since the anti-peptide sera are mono-specific with defined epitopes, they are valuable tools for future dissection of the mode of action of gpPH-20 in enzymatic function as well as the mediation of sperm-egg interaction. These experiments, together with the selection of mimotopes of conformation-dependent epitopes using phage-display technology, are important in the ultimate definition of PH-20 structure as it relates to the development of a peptide-based contraceptive vaccine.

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