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Molecular cloning of the human and monkey sperm surface protein PH-20

(fertilization/contraceptive vaccine)

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ABSTRACT **The guinea pig sperm surface protein PH-20 has an essential function in sperm adhesion to the zona pellucida of guinea pig eggs. Fully effective contraception has been achieved by immunizing either male or female guinea pigs with purified guinea pig PH-20. Here we report the isolation of human and cynomolgus monkey PH-20 cDNAs as a key step toward testing the function of primate PH-20 and the contraceptive efficacy of PH-20 immunization in primates. The deduced amino acid sequence of human PH-20 has 509 residues and is 59% identical with guinea pig PH-20, suggesting they may have a conserved function and immunogenicity. Southern blots show that there is a single PH-20 gene in the human genome and Northern blots of human testis poly(A)$^+$ RNA show a 2.4-kb message. Northern blots of tissues other than testis are negative for PH-20, indicating that human PH-20 is testis-specific.**

Sperm cells contain many kinds of potentially antigenic molecules. Certain infertility patients, both men and women, are subfertile or infertile because they produce anti-sperm antibodies (1, 2). Also, male and female animals immunized with whole sperm (or testis) extracts show some degree of infertility (3–7). Sperm antigens are therefore of great interest as target antigens for developing contraceptive vaccines. In previous work, we obtained fully effective contraception in male and female guinea pigs immunized with the guinea pig sperm protein PH-20 (8).

In guinea pigs, it has been shown that PH-20 has a required role in binding sperm to the egg zona pellucida in the early stages of fertilization (9). Guinea pig PH-20 (gpPH-20) is a glycosyl phosphatidylinositol (GPI)-anchored membrane protein with relative molecular mass of 64 kDa (10). gpPH-20 is present on the plasma membrane and inner acrosomal membrane. The plasma membrane population migrates to the inner acrosomal membrane after the acrosome reaction (9, 11). On acrosome-reacted sperm, gpPH-20 on the inner acrosomal membrane functions in sperm adhesion to the zona (9, 12).

In mammals adhesion of acrosome-intact sperm or acrosome-reacted sperm to the egg zona pellucida is a species-specific process (refs. 12–14; unpublished results). Sperm surface proteins active in adhesion to the zona in different mammalian species could be entirely distinct from each other or, at the other extreme, could be closely related with key changes that lead to the species specificity. The amino acid sequences of zona pellucida glycoproteins ZP-2 and ZP-3 are known to be conserved among mammals (15–19). The conserved structure of zona proteins is thought to be important for the structural integrity of the zona pellucida. However, it is O-linked carbohydrate on mouse ZP-3 that is the adhesion site for acrosome-intact mouse sperm (20) and this is the only identified zona adhesion target for sperm. Extensive species-to-species variation in zona protein carbohydrate structures could conceivably result in species-to-species variation in the identity of sperm adhesion proteins that bind to them.

Genomic Southern blots, probed with gpPH-20 cDNA, show that a PH-20 gene is present in a wide range of mammalian species, suggesting that PH-20 has some conserved features among mammals (21). In this study we cloned and sequenced cynomolgus monkey PH-20 (cPH-20) and human PH-20 (hPH-20)† and found that PH-20 has a conserved structure in rodents and primates. cPH-20 and hPH-20 were cloned from testis cDNAs and therefore may be expressed on sperm and have a similar function in mediating sperm–zona adhesion.

The clones for cPH-20 and hPH-20 will allow us to explore further the function of primate PH-20. These clones also open up the possibility of using PH-20 as an antigen for a human contraceptive vaccine. Expression and purification of recombinant monkey and hPH-20 will permit testing of the contraceptive potential of PH-20 immunization in primates.

MATERIALS AND METHODS

Library and Tissues. The human testis λgt11 library was the kind gift of Jose Millan (La Jolla Cancer Research Foundation, La Jolla, CA). Cynomolgus monkey testis was provided by Cathi VandeVoort and James Overstreet (University of California, Davis). Human testis was provided by Myron Walzak (Department of Urology, University of Connecticut Health Center). Other human tissues were obtained from National Disease Research Interchange (Philadelphia).

Cloning and Sequencing of hPH-20 cDNA. The H18 clone was isolated by plating the human testis cDNA library at a density of 3000 plaques per 90-mm plate. Duplicate filters were screened using as probe a mixture of two clones encoding full-length guinea pig PH-20 (21) and N-terminal mouse (M1) PH-20 (22) open reading frames. Hybridization was at 50°C overnight. The filters were washed three times at room temperature for 5 min with 2× SSC/0.1% SDS (1× SSC = 0.15 M sodium chloride/15 mM sodium citrate) and at 50°C once for 30 min with 2× SSC/0.1% SDS.

PCR Cloning of cPH-20 cDNA. Poly(A)$^+$ RNA was isolated from cynomolgus testis (21) and reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) using a primer from the sequence of the 3′ untranslated region (3′ UTR) of the hPH-20 clone H18 (5′-AAGCACTTTAAGATGG-3′). The resulting cDNA was used as template for PCR with two primers derived from the H18 sequence, 5′ primer (5′-gcgggatc-

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Abbreviations: GPI, glycosyl phosphatidylinositol; gpPH-20, guinea pig PH-20; mPH-20, mouse PH-20; cPH-20, cynomolgus monkey PH-20; hPH-20, human PH-20; UTR, untranslated region.

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L13780 and L13781).

Petitioner Merck, Ex. 1194, p. 10071
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00046

cgctagcTTTACAATGGGAGTGC-3′) and 3′ primer (5′-cgggatccgctagcGCGCAATTACAAACTCG-3′). Two or three random bases followed by *Bam*HI and *Nhe* I sites (bases shown in lowercase) were included at the 5′ ends of each PCR primer to allow subcloning of the PCR product. Conditions for PCR were those specified by the supplier for vent polymerase (New England Biolabs). Primer annealing was at 40°C for the first 5 cycles followed by 65°C for the next 25 cycles, extension was at 72°C for 1.5 min, and denaturation was at 94°C for 1 min.

Northern Blots. Poly(A)$^+$ RNA was prepared from human testis total RNA by oligo(dT)-cellulose chromatography (23) and was electrophoresed and then blotted onto a nitrocellulose membrane (Bio-Rad). Prehybridization and washing of the membrane were carried out as described (21). ^{32}P-labeled probe was prepared using H18 cDNA as template. To test for the expression of PH-20 mRNA in other tissues, total RNA was prepared from various human tissues and in some cases poly(A)$^+$ RNA was isolated. Samples were electrophoresed and transferred to nitrocellulose. ^{32}P-labeled probe was again prepared using H18 cDNA as template. After autoradiography of the membrane probed with H18, the nitrocellulose was washed with elution buffer (0.05× SSC/0.01 M EDTA/0.1% SDS) at 100°C for 15 min and reprobed with ^{32}P-labeled β-tubulin cDNA (insert of pMβ5 plasmid; gift of Joel Pachter, University of Connecticut Health Center). Hybridization conditions for the tubulin probe were the same as those used with the hPH-20 probe.

Southern Blots. Ten micrograms of genomic DNA isolated from human blood cells (24) was cut with *Eco*RI, electrophoresed, and transferred onto a Magnagraph nylon membrane (Micron Separations, Westboro, MA). The membrane was prehybridized and hybridized as described (21). The ^{32}P-labeled probe (H18 cDNA) was the same as used for the Northern blot.

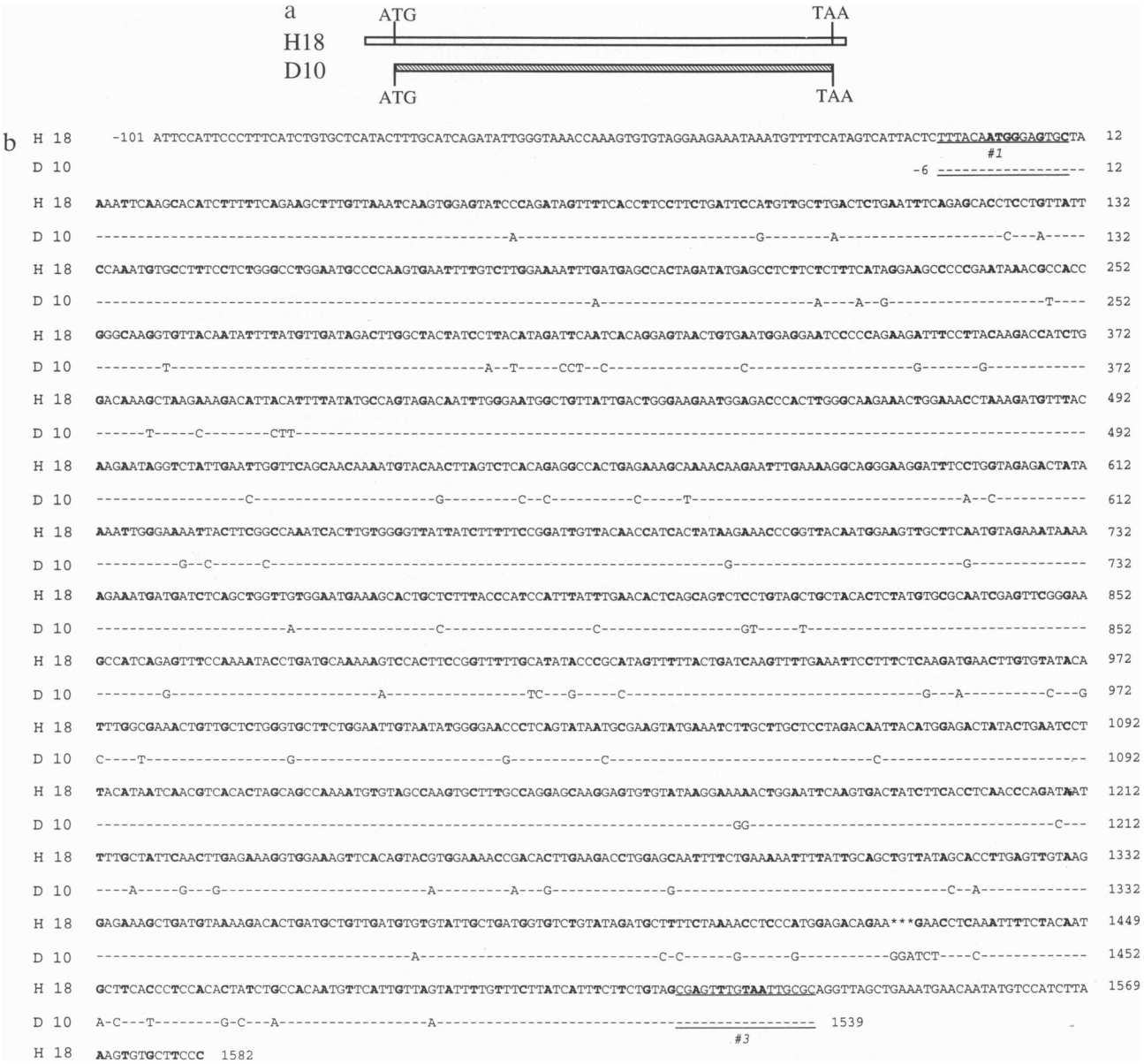

FIG. 1. Comparison of cDNA sequences of the human clone (H18) and the cynomologus monkey clone (D10). (*a*) Schematic map of H18 and D10 cDNAs. ATG is the first methionine in the open reading frame; TAA is the termination codon. The shaded region indicates the close sequence relationship between human and monkey. (*b*) Nucleotide sequences of H18 and D10 cDNAs. Dashes indicate bases identical to the corresponding base in the H18 sequence. Underlined in H18 are the sequences used to make the PCR primers 1 and 3 to generate D10. The corresponding primer sequences found in D10 are also underlined. Start codon (ATG), stop codon (TAA), and every first base in the codons are in boldface type. There is an *Eco*RI site (GAATTC) in H18 and in D10 (1176–1181).

Petitioner Merck, Ex. 1194, p. 10072
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00046

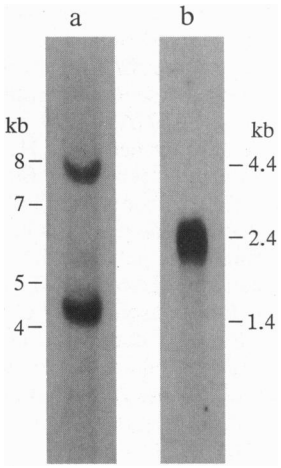

FIG. 2. (*a*) Southern blot of human genomic DNA using labeled H18 cDNA as probe. Human genomic DNA (10 μg) was digested with *Eco*RI. Size markers are at the left. (*b*) Northern blot of human testis poly(A)$^+$ RNA probed with ^{32}P-labeled H18, indicating the presence of a single 2.4-kb mRNA. Positions of RNA size markers are at the right.

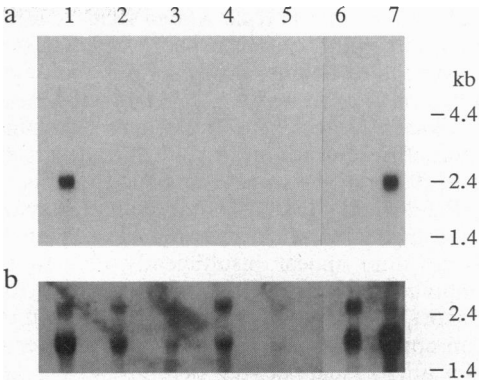

FIG. 3. Northern blot probed with PH-20 cDNA (*a*) or β-tubulin cDNA (*b*). (*a*) PH-20 mRNA expression determined by Northern analysis of total RNA prepared from testis (lane 1), uterus (lane 2), duodenum (lane 3), ovary (lane 4), and spleen (lane 5) or mRNA prepared from liver (lane 6) and testis (lane 7). Five micrograms of poly(A)$^+$ RNA or 20 μg of total RNA was used per lane. (*b*) The same blot, stripped and reprobed with β-tubulin cDNA.

RESULTS

Cloning and Sequencing of cDNA for hPH-20 and cPH-20. A λgt11 human testis cDNA library was screened with cDNA probes derived from gpPH-20 and mouse PH-20 (mPH-20) cDNA. Of 240,000 plaques screened, a positive clone was identified for hPH-20, H18 (Fig. 1). H18 contains 101 bp of 5′ UTR, 1530 bp of open reading frame, and 52 bp of 3′ UTR (Fig. 1*b*).

A cPH-20 cDNA clone, D10 (Fig. 1), was isolated by PCR using oligonucleotide primers derived from the sequence of H18. With primers 1 and 3 (Fig. 1*b*) a PCR product of 1547 bp was amplified from a reverse transcription product of cynomolgus testis mRNA. Three clones from three different PCRs were sequenced and two of these have identical sequences. The two identical sequences reveal that the cPH-20 cDNA coding region is very similar (94% identical nucleotides) to the hPH-20 cDNA coding region (Fig. 1*b*).

Southern and Northern Analysis. Southern blots of human genomic DNA show bands that hybridize with the H18 cDNA probe. When human genomic DNA is digested with *Eco*RI, which has a single recognition site in the cDNA sequence, two bands are found on the genomic blot (Fig. 2*a*). This suggests there is a single gene for hPH-20. Northern blot analysis of poly(A)$^+$ mRNA isolated from human testis indicates that the hPH-20 gene encodes one size mRNA (≈2.4 kb) in testis (Fig. 2*b*). Since the hPH-20 coding region is ≈1.5 kb, the full-length transcript apparently contains an additional ≈900 bp in its 5′ and 3′ UTRs.

The tissue pattern of PH-20 mRNA expression was examined by Northern analysis of poly(A)$^+$ or total RNA prepared from various human tissues (Fig. 3*a*). Among the tissues we examined, PH-20 mRNA was found only in testis and was undetectable in spleen, ovary, duodenum, uterus, and liver. The quality of the RNA samples from each tissue was tested by rehybridizing the same blot with a probe for β-tubulin mRNA. Two tubulin messages were detected in all tested tissues (Fig. 3*b*).

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H18   MGVLKFKHIFFRSFVKSSGVSQIVFTFLLIPCCLTLNFRAPPVIPNVPFLWAWNAPSEFC   60
D10   ---------------------------------------I----------------      60
GP    --AFT---S--G---EC---L-T--I------ -ADK ----L-----L--V----T--      59

H18   LGKFDEPLDMSLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITGVTVNGGIPQKISL   120
D10   ----N--------TLM------V--------------------LT-----H------V--   120
GP    I-GTNQ-----F--IV-T--K-I---SI-LY------------PH--AI-H--L--LMN-   119

H18   QDHLDKAKKDITFYMPVDNLGMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNVQLS   180
D10   ------S-Q--L-----------------------------------------------   180
GP    -Q--R-SRQ--L----T-SV-L------------T---R---I-R-K-----KS-HP-YN   179

H18   LTEATEKAKQEFEKAGKDFLVETIKLGKLLRPNHLWGYYLFPDCYNHHYKKPGYNGSCFN   240
D10   -PQ--D-------------ML------RS---------------------R-------D   240
GP    HSY-VAV--RD--RT--A-ML--L----S---SS----------------T-FT--N-D-H-PP   239

H18   VEIKRNDDLSWLWNESTALYPSIYLNTQ QSPVAATLYVRNRVREAIRVSKIPDAKSPLP   299
D10   ---------------------------- --V-V----------------------N---   299
GP    I-LQ--N--Q----D-------V--TSRVR-SQNGA-------H-S-----LM-D-N---   299

H18   VFAYTRIVFTDQVLKFLSQDELVYTFGETVALGASGIVIWGTLSIMRSMKSCLLLDNYME   359
D10   --V-A-L-----------RE---S-L---------------S---T-------------T---   359
GP    IYV-I-L-----TTT--EL-D--HSV--I-P--V---I---S--LT--LV--IG-E---K   359

H18   TILNPYIINVTLAAKMCSQVLCQEQGVCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRG   419
D10   ----------------------------------D-------------D-R---------H-   419
GP    GT-L--L----------G----KN--I-T--D--TNT-----AT--D-E-QQN---V-H-   419

H18   KPTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDVCIADGVCIDAFLK PPMETE E   477
D10   ---V----E-------------TN-------------------------S-- --V---GS   478
GP    --S----QE--KN-H----TNVA--DRL--HNVRS-N--T-NNI----V-NF-SLDDDD-   479

H18   PQIFYNASPSTLSATMFIVSI                LFLIISSVASL*              509
D10   -P----T-S--V-T-----N-                -----------*              510
GP    -P-TDDT-QNQD-ISDITS-APPSSHILPKDLSWC---LSIFSQHWKYLL*            529
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FIG. 4. Comparison of the deduced amino acid sequences of hPH-20 (H18), cPH-20 (D10), and gpPH-20 (gp) (21). Dashes indicate amino acids identical to the corresponding amino acid in the H18 sequence. Shaded areas represent conserved cysteines; potential glycosylation sites are in boldface type.

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Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00046

Deduced hPH-20 and cPH-20 Amino Acid Sequences and Comparison with gpPH-20. The primary structure of hPH-20 deduced from the H18 clone consists of 509 amino acids (Fig. 4). cPH-20, having an extra glycine residue near its C terminus (clone D10, residue 477), contains 510 amino acids. hPH-20 and cPH-20 differ in 53 residues and thus exhibit a high degree (90%) of amino acid sequence identity (Fig. 4).

Both cPH-20 and hPH-20 lack a potential cytoplasmic domain and their C-terminal hydrophobic sequences (residues 491 to stop) appear insufficiently long to serve as transmembrane anchors. These two features (no cytoplasmic domain, short C-terminal hydrophobic region) and the presence of an appropriate cleavage site (between Ser-490 and Ala-491 for hPH-20 and between Ser-491 and Thr-492 in the case of cPH-20) are likely to signal attachment of a GPI anchor (25).

The deduced amino acid sequences of hPH-20 and cPH-20 are typical for an integral membrane protein with a signal sequence and a sequence for membrane anchoring. After cleavage of a predicted hPH-20 signal sequence at Thr-35 (26) and the GPI-anchor signal sequence at Ser-490 (25), the calculated molecular mass of the mature hPH20 polypeptide is 51.8 kDa.

DISCUSSION

The results in the present study have implications for understanding the basic biology of PH-20 and for its development as an antigen for a contraceptive vaccine.

One unanswered question about the biology of PH-20 is how this protein reaches its location in the cell. gpPH-20 after being synthesized is delivered to the acrosomal membrane and plasma membrane (27). Two hypotheses have been proposed to explain the targeting (21). One hypothesis is that two different forms of the PH-20 protein are encoded; these two forms contain different targeting information so that one is targeted to the acrosomal membrane and the other to the plasma membrane. The second hypothesis is that only a single form of PH-20 protein is made and developmental changes in spermatogenic cells allow it to go first to the acrosomal membrane and subsequently to the plasma membrane (21). Previous work on gpPH-20 protein and various gpPH-20 cDNA clones has thus far revealed only a single form for gpPH-20 (8, 11, 21). Our current findings with hPH-20 cDNAs also are consistent with the second hypothesis. In the screening of the human testis cDNA library, we were able to find a second positive clone, termed H16. H16 possesses an open reading frame of 828 bp of which the first 780 bp is identical with the 3′ portion of H18 (from 706 to 1486 bp) and differs from H18 at the very end of the open reading frame and in the 3′ UTR. When human testis poly(A)$^+$ RNA was probed with an H16-specific sequence (H16's 3′ UTR), no testis transcript was detectable within the sensitivity of a Northern blot (data not shown). Positive controls were included to ensure the quality of the ^{32}P-labeled probe and the easy detection of the H18 transcript in the poly(A)$^+$ RNA on the same blot. Thus the H16 clone either may result from a variant hPH-20 message expressed at a much lower level than H18 or may be a cloning artifact.

A second uncertainty exists pertaining to what sperm proteins function in sperm–zona adhesion in different species. A large group of sperm proteins that are candidates for sperm adhesion proteins for the zona have been reported (reviewed in ref. 19). Since these proteins have thus far been identified and their potential adhesion function has been studied in a single species, it remains unclear if they are present on sperm of various mammalian species and have a similar or identical function in each species. We have now found that PH-20 can be cloned from mouse (22), monkey, and human testis and has a structure that is highly conserved (see below) with gpPH-20, which functions in guinea pig sperm–zona adhesion. These results suggest that at least this particular protein, PH-20, may be present on sperm and have a conserved function in fertilization in many mammals.

Lee and Vacquier (28) have raised the question of whether proteins involved in gamete recognition and adhesion will evolve more rapidly than proteins with other functions. Our data show that hPH-20 and cPH-20 are very homologous to each other with 94% nucleotide identity and 90% amino acid identity. This degree of evolutionary change in PH-20 is similar to some other proteins for which human and nonhuman primates' sequences have been determined. For in-

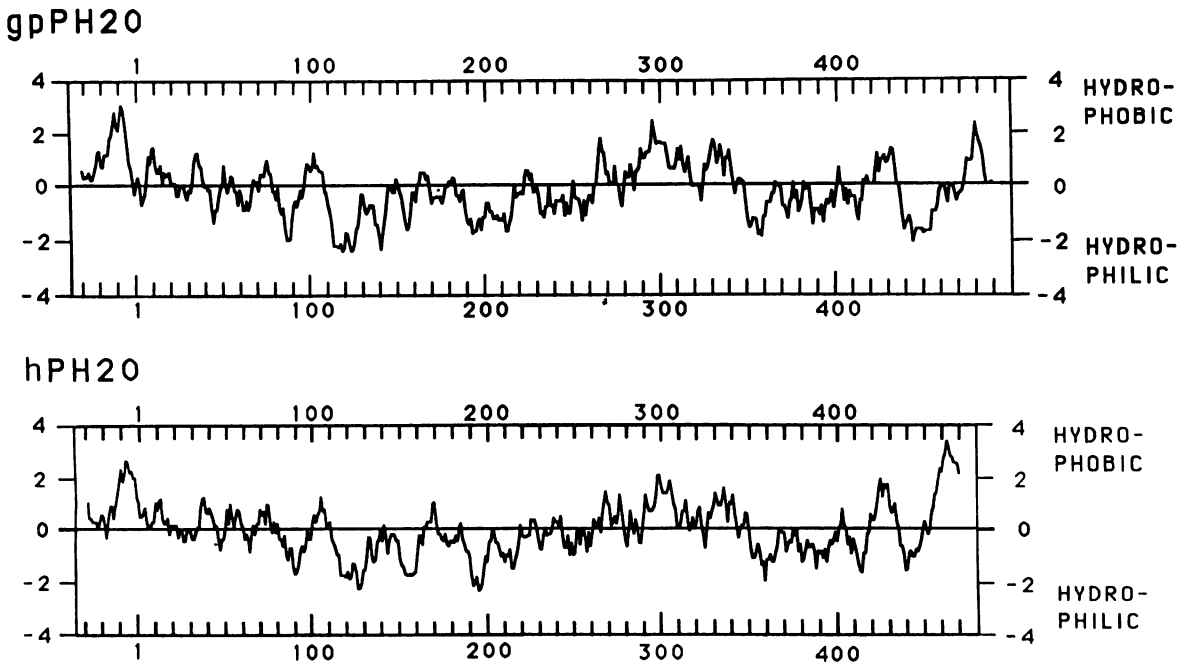

FIG. 5. Computer-generated hydropathy plots using the Kyte–Doolittle algorithm of gpPH-20 (*a*) and hPH-20 (*b*). Amino acid no. 1 is the first residue after the proposed signal sequence cleavage.

Petitioner Merck, Ex. 1194, p. 10074
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00046

stance, amino acid sequences for alcohol dehydrogenase between human and baboon are 97% identical (29, 30), human and baboon CD44 are 94% identical (31–33), human and rhesus monkey CD4 are 92% identical (34, 35), and human and cynomolgus monkey apolipoprotein C-II are 87% identical (36).

Although monkeys are evolutionarily close to human, human sperm do not bind to the zona pellucida of eggs from several different monkeys (37). Subtle alterations in PH-20 and/or other proteins functioning in sperm–zona adhesion may lead to the species specificity. Since amino acid residues of PH-20, altered between cynomolgus and human, do not cluster in one region, it is impossible to hypothesize which changed PH-20 residues might be related to species specificity.

Earlier studies have shown that gpPH-20 is a fully effective antigen for immunocontraception. When either male or female guinea pigs were immunized with purified gpPH-20, 100% effective contraception was obtained (8). The contraceptive effect in males is long-lasting and at least partially reversible; in females it is long-lasting and reversible (refs. 8 and 38; unpublished results). When sequences of hPH-20 and gpPH-20 (21) are compared, the similarity between them is obvious. There are seven potential sites for N-linked glycosylation in hPH-20 that are evenly distributed along the predicted extracellular domain (Fig. 4). gpPH-20 has six well-distributed N-linked glycosylation sites (21); four of them are in identical positions as in hPH-20. Furthermore, hydrophobicity plots (Fig. 5) of hPH-20 and gpPH-20 show very similar patterns. They both have a strongly hydrophobic signal sequence, a large internal hydrophobic domain (residues 290–350), and a short C-terminal hydrophobic region. As in hPH-20, in gpPH-20 the short C-terminal sequence is appropriate to signal GPI anchoring (21) and the gpPH-20 protein has been shown to have a GPI anchor (10). The amino acid sequence deduced from the hPH-20 cDNA clone has a high degree of homology with gpPH-20. They are 59% identical and 71% similar (conservative changes) and all 12 cysteines are conserved (Fig. 4). The sequence conservation suggests that the two proteins have similar overall structures and thus potentially conserved function and immunogenicity. Therefore, hPH-20 should be a good candidate antigen for developing a contraceptive vaccine for humans.

Although gpPH-20 has a high degree of sequence identity with mPH-20 (22) and hPH-20, polyclonal antibodies against gpPH-20 react only weakly with mouse sperm (unpublished results) or human sperm (38). Thus immunizing mice or humans with, for instance, gpPH-20 would be expected to have a weak (or no) effect on fertility. To test contraceptive efficacy in a certain species, PH-20 from that species must be used as immunogen, such as monkey PH-20 to test in monkey and mPH-20 to test in mouse, etc. The cloning and sequencing of cynomolgus PH-20 will enable us to test contraceptive efficacy in an animal model close to human.

One criterion for the selection of sperm vaccine antigens is that these antigens must be sperm-specific so that, when used as immunogens, they will not elicit autoimmune responses. With gpPH-20, we previously tested for its presence in various guinea pig tissues by radioimmunoassay and detected it only on spermatogenic cells and sperm (8). Here we examined the distribution in human tissues of hPH-20 mRNA expression and detected its presence only in testis. This finding again suggests the feasibility of developing hPH-20 as an antigen for a human contraceptive vaccine.

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Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00046