Identification of a Hyaluronic Acid (HA) Binding Domain in the PH-20 Protein That May Function in Cell Signaling

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ABSTRACT The macaque sperm surface protein PH-20 is a hyaluronidase, but it also interacts with hyaluronic acid (HA) to increase internal calcium ($[Ca^{2+}]_i$) in the sperm cell. A region of the PH-20 molecule, termed Peptide 2 (aa 205–235), has amino acid charge homology with other HA binding proteins. The Peptide 2 sequence was synthesized and two recombinant PH-20 proteins were developed, one containing the Peptide 2 region (G3, aa 143–510) and one without it (E12, aa 291–510). On Western blots, affinity-purified anti-Peptide 2 IgG recognized the 64 kDa band corresponding to PH-20 in acrosome intact sperm and, under reducing conditions, recognized the whole 67 kDa PH-20 and the endoproteolyzed N-terminal fragment of PH-20. HA conjugated to a photoaffinity substrate specifically bound to sperm surface PH-20. Indirect immunofluorescence demonstrated that Fab fragments of anti-Peptide 2 IgG bound to the head of live sperm. Biotinylated HA was bound by Peptide 2 and by sperm extracts in a microplate binding assay, and this binding was inhibited by Fab fragments of anti-Peptide 2 IgG. Biotinylated HA bound to the G3 protein and this binding was inhibited by anti-Peptide 2 Fab, but HA did not bind to the E12 protein. Fab fragments of anti-Peptide 2 IgG inhibited the increase in $[Ca^{2+}]_i$ induced in macaque sperm by HA. Our results suggest that the Peptide 2 region of PH-20 is involved in binding HA, which results in the cell signaling events related to the elevation of $[Ca^{2+}]_i$ during sperm penetration of the cumulus. *Mol. Reprod. Dev. 60: 542–552, 2001.*
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INTRODUCTION

Hyaluronan (hyaluronic acid, HA) is a simple linear polymer of repeating N-acetylglucosamine-glucuronic acid disaccharides. HA is highly conserved in mammals and ubiquitous in extracellular matrices (ECM). The mammalian oocyte is surrounded by ECM which sperm must traverse before fertilization (Yanagimachi, 1994). The HA-rich viscoelastic matrix of the cumulus oophorus is produced by granulosa cells in response to the ovulation-inducing LH surge (Cherr et al., 1990; Camaioni et al., 1996; Kobayashi et al., 1999; Salustri et al., 1999). The expanded cumulus mass has been shown to facilitate oviductal pick-up and transport of oocytes, exclude potential pathogens, reduce mechanical stress, stimulate sperm motility, improve sperm selection, enhance fertilization and promote embryo survival (Talbot, 1985; Cummins and Yanagimachi, 1986; Fetterolf et al., 1994; Ranganathan et al., 1994; Vandevoort et al., 1997; Familiari et al., 1998; Cherr et al., 1999; Hess et al., 1999; Talbot et al., 1999). Interactions between HA and sperm surface components during cumulus penetration can have direct effects on the functions of the sperm flagellum (Kornovski et al., 1994) and the acrosome (Cherr et al., 1999) to maximize the likelihood of fertilization. HA is thought to initiate signaling in many cell types (Bourguignon et al., 1993; Entwistle et al., 1996; Rao et al., 1997). Such HA-induced cell signaling may be involved in cell locomotion, angiogenesis, wound healing, tumor progression, and embryogenesis (Delpech et al., 1997; Chen and Abatangelo, 1999; Lokeshwar et al., 1999; Madan et al., 1999; Camenisch et al., 2000; Toole, 2000). Intense investigation is now in progress to identify the cell surface proteins that interact with HA and regulate its signal transduction activity. (Bourguignon et al., 1993; Aruffo, 1996; Fitzgerald et al., 2000; Oliferenko et al., 2000).

Hyaladherins (HA binding proteins, HABP) belong to the link protein superfamily and include ECM proteins (aggrecan, versican, IαI hyaluronectin), as well as cell surface receptors that may be involved in signaling (CD44, RHAMM, tumor necrosis factor-stimulated gene 6,TSG-6) (Toole, 1990; Neame and Barry, 1993; Iozzo and Murdoch, 1996; Cheung et al., 1999; Knudson et al., 1999). The HA binding domains of a number of

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these cell surface proteins have been identified and all appear to include one or more regions of basic amino acids. CD44 and TSG-6 appear to utilize very similar HA binding motifs, while RHAMM has been shown to rely on a B(X7)B alignment in which two basic amino acids are separated by seven neutral or non-acidic amino acids (Yang et al., 1994; Bajorath et al., 1998). Post-translational modifications of CD44, including glycosylation give rise to numerous isoforms that regulate HA binding capability (Sy et al., 1997; Borland et al., 1998; Greenfield et al., 1999). There is also evidence suggesting that the size of HA molecule may determine the signaling response in target cells. For example, binding of high molecular weight polymers of HA to CD44 isoforms facilitates cell adhesion, while binding to HA fragments results in activation of cell signaling events (Aruffo, 1996).

In both human and macaque sperm, HA induces an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) through an interaction with the glycosylinositol phosphate (GPI)-anchored sperm membrane protein, PH-20 (Sabeur et al., 1998; Cherr et al., 1999). It has been proposed that the binding of HA in the cumulus ECM to sperm surface PH-20 results in aggregation of the PH-20 protein, causing this signaling event (Cherr et al., 1999). The increase in $[Ca^{2+}]_i$ is not sufficient to trigger the acrosome reaction, but may facilitate induction of the acrosome reaction after sperm binding to the zona pellucida (Vandevoort et al., 1997). PH-20 is the mammalian sperm hyaluronidase (Gmachl and Kreil, 1993; Lin et al., 1994), but a distinct HA binding domain may exist which is separate from the hyaluronidase domain. Gacesa et al. (1994)identified a potential HA binding domain that is within one of the catalytic sites of PH-20 and is similar to the HA binding domain described for RHAMM (Yang et al., 1994; Yudin et al., 2001).

We have identified two regions of the macaque PH-20 molecule that are the catalytic sites responsible for hyaluronidase activity (amino acids 142–172 and 277–297; termed "Peptide 1" and "Peptide 3," respectively) (Yudin et al., 2001). In the present study, we have investigated a different putative HA-binding domain of macaque sperm PH-20 and its function in cell signaling. This domain, termed "Peptide 2" (amino acids 205–235), includes alternating basic amino acids (lysine, arginine) and neutral amino acids characteristic of a heparin-binding motif (Ferran et al., 1992; Sobel et al., 1992). Our findings demonstrate that the synthetic peptide corresponding to the Peptide 2 domain is a potent HA binding peptide, and that a recombinant PH-20 protein containing the Peptide 2 domain binds HA, while one that lacks that domain does not. We also show that the Peptide 2 domain mediates the increase in sperm $[Ca^{2+}]_i$ that is induced by HA. Taken together, the results of the present study and our previous studies (Yudin et al., 2001) provide direct evidence that the PH-20 protein has two distinct functional domains for hyaluronidase activity and HA-induced cell signaling.

MATERIALS AND METHODS

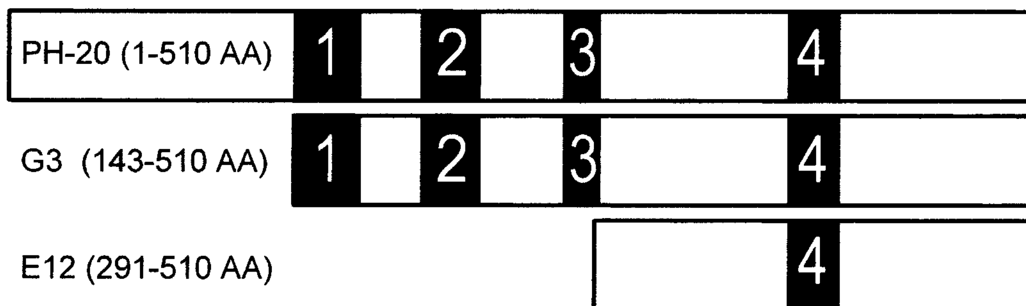
Reagents

Fluo-3 AM and Pluronic F-127 were obtained from Molecular Probes (Eugene, OR). Fluorescein isothiocyanate hydrazide (FITC), protein A agarose, SuperSignal West Pico Chemiluminescent Substrate, sulfosuccinimidyl [2-6-(biotinamido)-2-(p-azidobenzamido)-hexanoamido]ethyl-1,3′-dithiopropionate (SBED), a protease inhibitor cocktail (4-(2-aminoethyl)-benzenesulfonyl fluoride, pepstatin A, trans-exposy-succinyl-L-leucylamido(4-guanidino)butane, bestatin, leupeptin and aprotinin), biotin-LC-hydrazide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and a Fab fragment purification kit were purchased from Pierce Scientific (Rockford, IL). Tris, glycine, nitrocellulose membranes, and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad Laboratories (Hercules, CA). Polyacrylamide gels were obtained from Fisher Scientific (Pittsburgh, PA). HABP was obtained from US Biological (Swampscott, MA). All other chemicals and reagents were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO), unless stated otherwise.

Antibodies to PH-20 and Synthetic Peptides

Recombinant cynomolgus macaque PH-20 (r-PH-20) was generated according to Lin et al. and was a gift from Dr. Paul Primakoff and Dr. Diana Myles. Two New Zealand white rabbits were immunized with 50 μg of r-PH-20 and Freund's complete adjuvant (first injection) or Freund's incomplete adjuvant (second and third injections). Rabbits were exsanguinated approximately 2 months after the initial injection. IgG was isolated from whole serum using Protein A agarose beads followed by elution with glycine buffer (pH 3.5), dialysis against phosphate buffered saline (PBS), and concentration and desalting with Centricon (Amicon, Danvers. MA). Fab fragments were prepared from whole IgG using a Fab fragment kit.

Four synthetic peptides and the respective anti-peptide antibodies were generated by Zymed Laboratories (South San Francisco, CA). The location of these peptides in the PH-20 protein is shown in Figure 1. Peptide 1 is the putative acid/base catalyst site for the hyaluronidase activity of PH-20 (Yudin et al., 2001). Peptide 2 represents a hydrophilic region with characteristic repeating basic amino acids that could serve as an HA binding site (Table 1). Peptide 3 is the putative nucleophile site for hyaluronidase activity of PH-20 (Yudin et al., 2001). The amino acid sequences represented by Peptides 1, 2 and 3 are contained within the N-terminal region of the PH-20 molecule. Peptide 4 is located in the C-terminal region and was generated as a negative control since it has little homology to other hyaluronidases or HA binding proteins. Rabbits were immunized with the synthetic peptides coupled to Keyhole Limpet Hemocyanin and exsanguinated approximately 2 months after the initial injection. Whole rabbit serum, IgG, specific anti-peptide IgGs



Peptide 1: (142)AVIDWEEWRPTWARNWKPKDVYKNRSIELV(172)
Peptide 2: (205)KLGRSLRPNHLWGYYLFPDCYNHHYRKPGYN(235)
Peptide 3: (277)YVRNRVREAIRVSKIPDAKN(297)
Peptide 4: (387)CIRKDWNSSDYLHLNPDNFDIRLEK(412)

Fig. 1. Schematic representation of the PH-20 protein indicating the relative locations of the synthetic peptides 1–4. The relationship of the recombinant proteins G3 and E12 to the PH-20 protein are also shown.

and Fab fragments of the anti-peptide IgGs were furnished by Zymed.

Expression and Purification of Recombinant PH-20 Proteins

Recombinant macaque PH-20 proteins were expressed in *E. coli* and purified as described by Deng et al. (in press). Briefly, total RNAs from cynomolgus macaque testis tissue were extracted and macaque PH-20 cDNA was synthesized by reverse transcription-coupled polymerase chain reaction (RT-PCR). For the G3 recombinant protein (encoding amino acids 143–510, Fig. 1), the primers for PCR amplification were: 5′-GGCTGTTATTGACTGGGAAG-3′ (forward, nucleotides 432–451) and 5′-ACAAACTCGCTACAGAAATG-3′ (reverse, nucleotides 1515–1536). For the E12 recombinant protein (encoding amino acids 291–510, Fig. 1), the primers were: 5′-AATACCTGATGCAAAAAATCC-3′ (forward, nucleotides 876–896) and 5′-ACAAACTC-GCTACAGAAATG-3′ (reverse, nucleotides 1515–1536). The PCR products were cloned into a pTriEx™–1 vector (Novagen, Madison, WI) and transformed into NovaBlue Singles™ competent cells (Novagen).

Following culture of the bacterial constructs, recombinant protein expression was induced by addition of a tiered λCE6 phage stock. The bacterial cells were lysed, the inclusion bodies composed primarily of the insoluble recombinant proteins were purified, and the recombinant proteins were solubilized in PBS containing 5% SDS. The majority of the SDS was removed by dialysis and the authenticity of the recombinant proteins was confirmed by immunoblotting.

Semen Collection and Sperm Capacitation

Semen samples were collected from male cynomolgus macaques by electroejaculation as per Sarason et al. (1991). Animals were housed at the California Regional Primate Research Center in compliance with the Federal Animal Welfare Act and the National Institutes of Health Guidelines for Care and Use of Laboratory Animals. Semen samples were diluted with 5 ml of modified Biggers, Whitten and Whittingham (BWW) medium (Irvine Scientific, Santa Ana, CA) with 21 mM HEPES buffer (pH 7.4). Samples were allowed to settle for 30 min and the upper 4 ml were removed and diluted to 8 ml with BWW-HEPES containing 3 mg/ml bovine serum albumin (BSA). Sperm suspensions were centrifuged at 300 × g for 10 min, resuspended in 5 ml of the BWW-HEPES-BSA and again centrifuged at 300 × g for 10 min. Sperm were resuspended in fresh BWW-HEPES-BSA to a concentration of 20×10^6/ml. After incubation for 24 hr at room temperature (RT), sperm were washed again as previously described and layered under 3 ml BWW-BSA containing 35.7 mM

TABLE 1. Putative HA-Binding Domains Within the Peptide 2 Region in Hyaluronidases and the Similarity to the Heparin Binding Protein

Monkey	[K] L G [R] S L [R] P N H L W G [Y Y] L F P D C Y N H H Y R K P G Y N G S C F D
	└──── Peptide 2 Region ────┘ (K through N)
Human	[K] L G [K] L L [R] .... [Y Y] .... C C
Bovine	[K] L G [K] L L [R] .... [Y Y] .... C C
Guinea pig	[K] A A [K] R L [R] .... [Y Y] .... C C
Bee venom	[K] A A [K] R M [R] .... [Y Y] .... C C
Heparin	[R] G L [R] H [R] L G [R] ....

sodium bicarbonate (HCO_3). After 1 hr of incubation at 37°C in a 5% CO_2 incubator, sperm were removed from the top 2 ml of medium and incubated for an additional 1 hr at 37°C. Sperm capacitation was completed by adding 1 mM each caffeine and N^6,2′-0-dibutyryladenosine 3′: 5′-cyclic monophosphate sodium salt (dbcAMP) to the sperm suspension and incubating for 30 min at 37°C.

Extraction of Sperm Samples

Capacitated sperm were washed once in Tris buffered saline (TBS) containing a protease inhibitor cocktail, incubated in TBS/protease inhibitor for 15 min at RT, then at 4°C for 5 min. Sperm were extracted in TBS containing protease inhibitor cocktail, 1% triton-X-100, and 30 mM n-octyl β-D-glucopyranoside (OG) for 1 hr at 4°C. Sperm were centrifuged at 3500 × g for 10 min, passed through a 0.22 μm filter (Fisher) and the supernatant was aliquoted and stored at −70°C.

Photoaffinity Crosslinking of HA to Sperm

For photoaffinity crosslinking experiments, HA was first modified to add amino groups as per Hermanson (1996), as follows. HA (4 mg/ml) was dissolved in 50 mM boric acid, pH 5.2, and ethylenediamine dihydrochloride added at a ratio of 20:1 by weight. EDC (final concentration 100 mM) was slowly added to the mixture, and the pH was maintained at 5.2. The solution was allowed to react for 1 hr at RT, quenched with β-mercaptoethanol (BME) at a 10:1 ratio (v/v), and dialyzed against PBS with 3 changes. The modified HA (1 ml) was then conjugated to SBED dissolved in DMSO according to Pierce (Yannariello-Brown, 1996). The mixture was incubated in the dark for 30 min at RT, followed by dialysis against PBS. The conjugated HA-SBED (200 μg/ml) was incubated with capacitated, acrosome-intact sperm for 10 min at RT in the dark, then photoactivated by exposure to UV light (365 nm) at a distance of 5 cm for 20 min. Sperm were centrifuged through 10% Ficoll (1 ×) and PBS (1 ×) at 990 × g for 5 min, pelleted at 990 × g for 10 min, and solubilized in reducing buffer for electrophoresis and Western blot analysis.

Immunolocalization

Capacitated, acrosome intact sperm were incubated for 30 min with Fab fragments of anti-Peptide 2 IgG (10 μg/ml). The sperm were centrifuged and resuspended in 0.8% paraformaldehyde for 15 min. Sperm were centrifuged and blocked (3 ×) in DPBS containing 1% BSA and 0.2% NaN_3, then incubated in 1:10 (V/V) goat anti-rabbit Fab conjugated with FITC (E-Y Laboratories, Hercules, CA) in DPBS/BSA. Sperm were washed and suspended in a solution of glycerol:1% paraformaldehyde (1:1). Sperm were visualized by fluorescence microscopy at excitation 490 nm (Sutter Instruments Lambda-10-2, Novato, CA) and emission 530 nm (interference filter). Images were captured using an MTI-CCD Dage camera interfaced to a Scion framegrabber through NIH Image 1.69 on a Macintosh computer.

Immunoblotting

Extracts of capacitated, acrosome intact sperm, extracts of acrosome reacted sperm, r-PH-20, G3, and E12 were solubilized with or without 5% BME in buffer (0.3 M Tris, 5% SDS, and 50% glycerol, pH 6.8) and boiled for 3 min. Proteins were electrophoresed using precast 10% Tris-glycine gels, followed by transfer to nitrocellulose membranes. The membranes were blocked overnight at 4°C in 10 mM Tris-HCl, 100 mM NaCl, 5% non-fat dry milk, and 0.1% gelatin, pH 7.5. The membranes were incubated with rabbit anti-peptide IgGs or anti-r-PH-20 IgG, washed several times in TBS containing 0.1% Tween-20 (TTBS), and incubated with goat anti-rabbit IgG-alkaline phosphatase (Bio-Rad). Following several washes in TTBS, the bands were visualized using 1-step NBT/BCIP (Pierce). Sperm-SBED extracts were probed with goat anti-biotin, washed several times in TTBS, and visualized using chemiluminescence.

HA Binding Assay

HA was biotinylated as per Yang et al. (1995). HA (4 mg/ml) was dissolved in 50 mM boric acid, pH 5.2, and biotin-LC-hydrazide was added at a ratio of 20:1 by weight. EDC (final concentration 100 mM) was slowly added to the mixture and the pH was maintained at 5.2. The solution was gently mixed for 16 hr at RT. The reaction was stopped by dialyzing against 500 mM sodium acetate, pH 4.0, for 4 hr at RT, followed by dialysis against PBS, pH 7.4, for 16 hr with two changes of buffer. Aliquots of biotinylated HA were stored at −70°C until use.

Binding of HA was assayed as per Dolzhenko et al. (1994) with modifications. Microtiter plate wells were coated with sperm extracts, Peptides 1–4 (10 μg/ml), or the recombinant proteins G3 and E12 (50 μM) dissolved in binding buffer (0.05 M citrate-phosphate buffer at pH 6.0). Plates were incubated for 15 hr at 4°C. Wells were washed twice with PBS containing 0.05% Tween 20 (PBS-T), and blocked for 1 hr with binding buffer containing 1% BSA at RT. Wells containing the sperm extracts, G3 or E12 were incubated for 2 hr at RT with Fab fragments of anti-peptide IgG or anti-r-PH-20 IgG (10 μg/ml) in PBS (pH 7.2). Wells containing Peptides 1–4 were incubated for 2 hr with their corresponding anti-peptide IgG Fabs (10 μg/ml). Wells were washed 4 times in PBS-T, followed by the addition of biotinylated HA or non-biotinylated HA (10 μg/ml) to the appropriate wells, and incubated for 2 hr at RT. Quercetin (50 μM) was included in all incubations of sperm extracts to inhibit hyaluronidase activity (Li et al., 1997b). Wells were washed 4 times in PBS-T, then incubated with goat anti-biotin conjugated to horseradish peroxidase for 1 hr at RT. Wells were washed 6 times in PBS-T and the enzyme reaction developed with 0.4 mg/ml *o*-phenylenediamine dihydryochloride) and 0.012% hydrogen peroxide in 0.05 M phosphate-citrate buffer (pH 5.0). The reaction was stopped by the addition of 50 μl of 2 M HCl and the absorbance at

490 nm measured on a microplate reader (Bio-Rad). Binding of biotinylated HA to sperm extracts, peptides, or recombinant proteins was compared to the binding response of a commercial HABP which was assigned a value of 1. In the case of the recombinant proteins, there was an elevated background signal due to non-specific binding of biotinylated HA to the histidine tag. An irrelevant histidine-tagged recombinant protein was included in the assays as a control, and the G3 or E12 signals were subtracted from the control signal. Non-biotinylated HA (20 μg/ml) and BSA (10 μg/ml) were used as negative controls.

Measurement of Intracellular Calcium ($[Ca^{2+}]_i$)

Sperm (1×10^7) were loaded with Fluo-3-AM (2.5 μM) from a 1 mM stock solution in DMSO/Pluronic F-127 for 2 hr at RT in the dark (Yudin et al., 1998; Cherr et al., 1999). Following loading, sperm were layered over 10% Ficoll in BWW and centrifuged for 10 min at $750 \times g$. Sperm were then resuspended and centrifuged in 12 ml BWW at $600 \times g$ for 6 min. Loaded sperm were layered under 3 ml of BWW and incubated for 60 min in a 37°C CO_2 incubator. Motile sperm from the upper 2 ml of media were incubated for an additional 60 min. Aliquots of sperm (500 μl) were treated with caffeine and dbcAMP as described above for 30 min prior to fluorometry. Changes in $[Ca^{2+}]_i$ in response to the addition of HA (100–200 μg) were measured in Fluo-3 loaded, capacitated sperm ($1–5 \times 10^5$) suspended in stirred methacrylate cuvettes at 37°C in a spectrofluorometer (Photon Technology International) at excitation 502 nm and emission 536 nm. Maximum fluorescence (R_{max}) following the addition of 50 μM (final) digitonin, and minimum fluorescence (R_{min}) following the addition of Tris-buffered EGTA (5 mM final) were measured for each experiment as per Grynkiewicz et al. (1985). Each experiment consisted of the addition of human umbilical cord HA to sperm (final concentration of 100–200 μg/ml) with or without pretreatment with IgG Fab fragments of anti-Peptide 1, anti-Peptide 2, or anti-r- PH-20 (10 μg/ml) 10 min prior to measurements. The relative difference in fluorescence between sperm undergoing these treatments was compared within each experiment ($n = 6$).

Statistical Analysis

Results are expressed as mean ± standard error. Data were analyzed using ANOVA (SPSS Scientific, Chicago, IL) followed by Tukey's test for differences among means. Significance was determined at values of $P < 0.05$. Experiments were conducted a minimum of three times.

RESULTS

Immunoblotting of Whole Sperm Extracts and Recombinant PH-20 Proteins With Anti-Peptide IgGs

Anti-Peptide 2 IgG and anti-Peptide 4 IgG recognized a single band at 64 kDa in extracts of non-reduced acrosome intact sperm (Fig. 2, lanes 1 and 2). When extracts of acrosome reacted sperm were analyzed under reducing conditions, anti-Peptide 2 IgG recognized bands at 67 and 44 kDa (Fig. 2, lane 3) and anti-Peptide 4 IgG recognized bands at 67, 32, and 30 kDa (Fig. 2, lane 4). We have shown previously that anti-r-PH-20 IgG recognizes a single band at 64 kDa in non-reduced acrosome intact sperm, and three bands at 67, 44, and 30 kDa in acrosome reacted sperm under reducing conditions (Yudin et al., 2001). We also showed that antibodies to Peptides 1 and 3 labeled the 64 kDa band in nonreduced acrosome intact sperm, and recognized the 67 and 44 kDa bands in acrosome reacted sperm under reducing conditions. The lower molecular weight forms have been attributed to the endoproteolytic cleavage of the PH-20 protein into two fragments which are disulfide linked (Primakoff et al., 1988). The higher molecular weight fragment (44 kDa) is the n-terminal fragment. When the recombinant PH-20 protein G3 (corresponding to amino acids 143–510, Fig. 1) was probed with antibodies to Peptides 1, 2, 3, and 4, a single band at 50 kDa was observed (Fig. 3A, lanes 1, 3, 5, 7). The recombinant protein E12 (corresponding to amino acids 291–510, Fig. 1) was recognized only by anti-Peptide 4 IgG, with a single band observed at 36 kDa (Fig. 3A, lane 8).

Immunolabeling of Sperm

We have previously shown that Fab fragments of IgG of anti-r-PH-20 and anti-Peptide 1, label the entire head of acrosome intact sperm, while Fab fragments of IgG from anti-peptide 3 primarily label the acrosomal region of the sperm head (Yudin et al., 2001). Here, we show that Fab fragments of anti-Peptide 2 IgG also label the acrosomal region of capacitated, acrosome intact sperm (Fig. 3B).

Binding of Biotinylated HA to Sperm Surface Proteins

We have previously shown that FITC-conjugated HA binds to the head region of capacitated, acrosome intact sperm (Cherr et al., 1999). In this study, live sperm were incubated with an SBED-HA conjugate and subsequently exposed to UV light resulting in the transfer of biotin to a putative HA binding sperm surface protein. Immunoblots of solubilized sperm probed with anti-biotin revealed a single band at 67 kDa (Fig. 4, lane 3). This band was also recognized by anti-r-PH-20 IgG (Fig. 4, lane 2), and is the correct molecular weight for reduced, nonendoproteolyzed PH-20 (Cherr et al., 1996).

HA Binding to Sperm Extracts, Synthetic Peptides, and Recombinant PH-20 Proteins

We utilized a microtiter plate assay to assess binding of biotinylated HA to sperm extracts (Fig. 5A), synthetic peptide regions of PH-20 (Fig. 5B), recombinant PH-20 proteins (Fig. 5C), or a commercial HABP. Biotinylated HA bound to the commercial HABP with a high degree of specificity as indicated by lack of

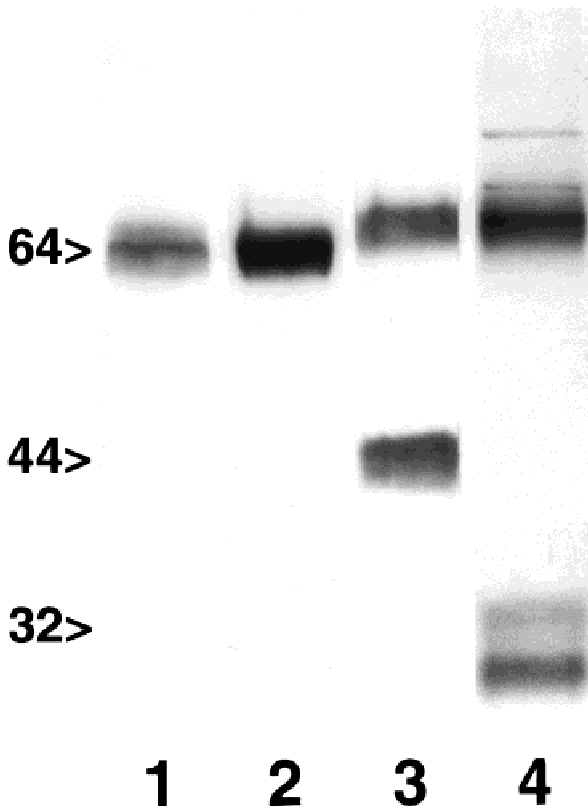


Fig. 2. Immunoblots of acrosome intact and acrosome reacted sperm probed with anti-peptide IgGs. Anti-Peptide 2 IgG (**lane 1**) and anti-Peptide 4 IgG (**lane 2**) recognize a single band at 64 kDa in acrosome intact sperm under non-reducing conditions. Under reducing conditions, anti-Peptide 2 IgG recognizes two bands at 67 kDa and 44 kDa (**lane 3**), while anti-Peptide 4 recognizes three bands at 67 kDa, 32 and 30 kDa (**lane 4**).

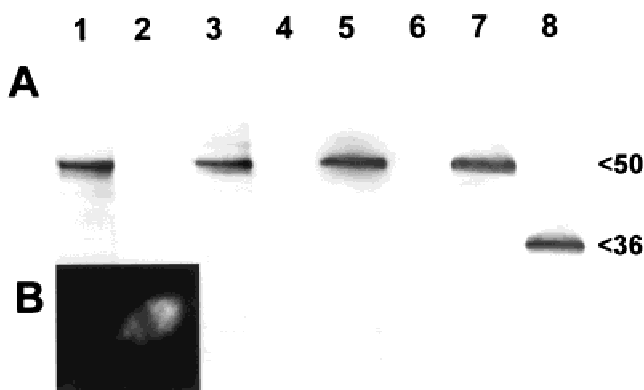


Fig. 3. A: Immunoblots of the recombinant PH-20 proteins G3 (**lanes 1**,**3**,**5**,**7**) and E12 (**lanes 2**, **4**, **6**, **8**) probed with anti-Peptide 1 IgG (lanes 1, 2), anti-Peptide 2 IgG (lanes 3, 4), anti-Peptide 3 IgG (lanes 5, 6) and anti-Peptide 4 IgG (lanes 7, 8). All four IgGs recognize a band at 50 kDa in lanes containing G3 (corresponding to amino acids 143–510). E12 (corresponding to amino acids 291–510) is not recognized by anti-Peptide IgGs 1, 2 or 3 (lanes 2, 4, 6), but a band at 36 KDa is recognized when E12 is probed with anti-Peptide 4 IgG (lane 8). **B:** Fluorescence microgrograph of live, capacitated acrosome intact sperm immunolabeled with anti-Peptide 2 Fab fragments showing labeling over the sperm head.

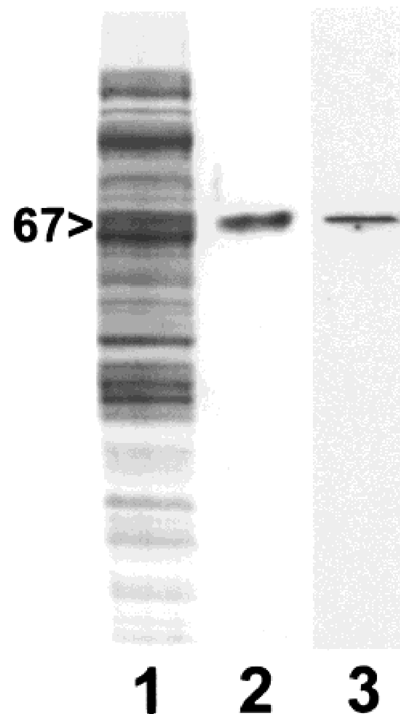


Fig. 4. Immunoblot of sperm surface proteins labeled with the photoactivatable biotinylated probe SBED conjugated to HA. **Lane 1:** Silver stained gel of sperm extract for total sperm proteins. **Lane 2:** Extract of live sperm incubated with anti-r-PH-20 IgG indicating a single band at 67 kDa. **Lane 3:** Extract of live sperm incubated with SBED-HA, followed by UV exposure showing a single band at 67 kDa when probed with anti-biotin.

binding to BSA (negative control) and an absence of signal when unlabeled HA was tested (data not shown). Although binding efficiency was enhanced at pH 5.0, the inhibitory effects of Fab fragments on binding were not observed at pH 5.0 because IgG Fab fragments bind poorly to antigen at this pH. Therefore, pH 6.0 was selected for both antibody inhibition studies and binding. Biotinylated HA binding to sperm extracts, synthetic peptides, and recombinant PH-20 proteins was always lower than the binding to commercial HABP (data not shown), and the experimental data were normalized to a binding response of 1 for the commercial HABP, which was always included as an internal standard.

Binding of biotinylated HA to sperm extracts was inhibited significantly by Fab fragments of anti-Peptide 2 IgG (70.6% inhibition) and anti-r-PH-20 IgG (70.3% inhibition) (Fig. 5A). While a slight decrease in binding was observed with Fab fragments of anti-Peptide 1 and 3 IgG, this inhibition was not significantly different from controls. Significantly more of biotinylated HA was bound to Peptide 2 than to any other synthetic peptide, and this binding was inhibited by pre-incubation with Fab fragments of anti-Peptide 2 IgG (73.0% inhibition, Fig. 5B). Incubation with Fab fragments of the other anti-peptide IgGs did not inhibit binding of biotinylated HA to Peptide 2, as illustrated by the data for anti-Peptide 1 IgG (Fig. 5B). The recombinant

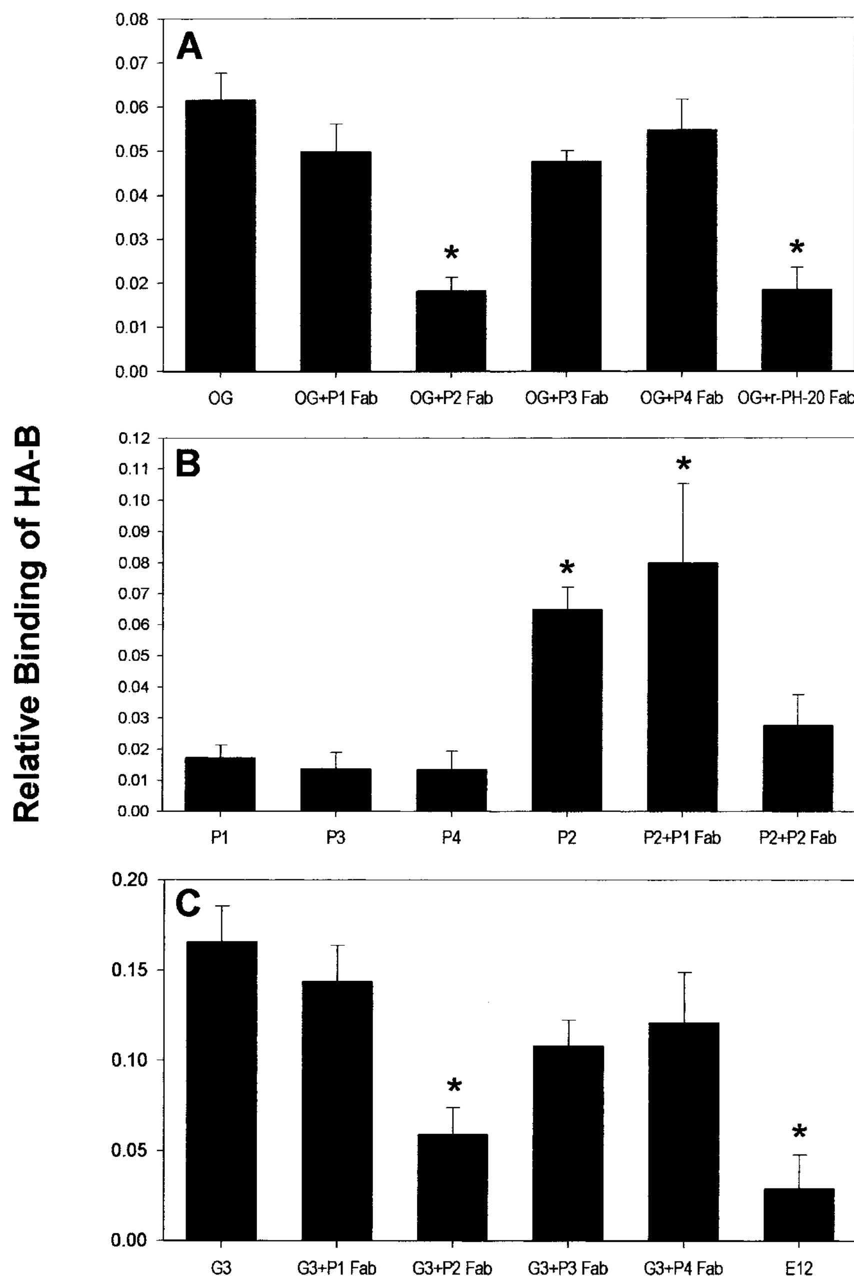


Fig. 5. Binding of biotinylated HA to sperm extracts (**A**), synthetic PH-20 peptides (**B**), or recombinant PH-20 proteins (**C**) and the effects of anti-peptide IgG Fab fragments or anti-r-PH-20 Fab fragments on binding. All binding responses were normalized to a commercial HA binding protein response of 1. No binding was detected using BSA as a negative control (not shown). Asterisks indicate significantly different binding at the $P < 0.05$ level.

protein G3 had much greater degree of binding than the E12 protein (Fig. 5C). Binding of biotinylated HA to G3 was significantly inhibited by Fab fragments of anti-Peptide 2 IgG (58.7% inhibition), but not anti-Peptide 1, 3 or 4 (Fig. 5C).

Effects of HA on Intracellular Calcium

We have previously shown that HA induces a 2-3 fold increase in $[Ca^{2+}]_i$, while Fab fragments of anti-r-PH-20 IgG inhibit this response (Cherr et al., 1999). In this study, pre-treatment of sperm with Fab fragments of anti-Peptide 2 IgG significantly inhibited the HA-induced increase in $[Ca^{2+}]_i$ (46.2% inhibition), which was comparable to the effect of anti-r-PH-20 Fab (45.8% inhibition), while pre-treatment with Fab fragments of anti-Peptide 1 IgG had no effect on the $[Ca^{2+}]_i$ increase (Fig. 6).

DISCUSSION

PH-20 is a GPI-anchored sperm protein that has been identified as the sperm surface hyaluronidase, with a high degree of homology to venom hyaluronidases

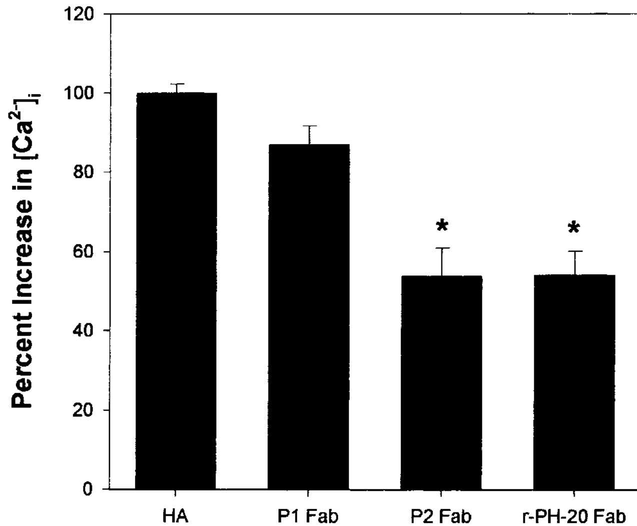


Fig. 6. The effect of Fab fragments of anti-Peptide 1 and 2 IgGs or anti-r-PH-20 IgG on the HA-induced increase in $[Ca^{2+}]_i$. Fluo-3 loaded sperm were exposed to 100 µg/ml HA and the fluorescence intensity measured in a fluorometer. Pre-treatment of sperm with Fab fragments of anti-Peptide 2 IgG and anti-r-PH-20 IgG inhibited the increase in $[Ca^{2+}]_i$ by 46.2 and 45.8% respectively. Fab fragments of anti-Peptide 1 IgG did not inhibit the increase. Asterisks indicate significance at the $P < 0.05$ level.

(Gmachl and Kreil, 1993; Frost et al., 1996). During sperm-egg interaction plasma membrane PH-20 has at least two functions in macaque sperm; its hyaluronidase activity depolymerizes the cumulus ECM thereby facilitating sperm penetration (Cherr et al., 1996; Li et al., 1997a; Meyers et al., 1997; Yudin et al., 2001), and through binding of HA, it initiates an intracellular signal that elevates $[Ca^{2+}]_i$ (Yudin et al., 1998; Cherr et al., 1999). We have synthesized peptides corresponding to the putative active sites for PH-20 hyaluronidase activity (Peptides 1 and 3; Yudin et al., 2001), to an additional putative HA binding site of the PH-20 molecule (Peptide 2; this study), and to a surface-exposed domain in the carboxyl region of the molecule (Peptide 4), which served as a negative control. Fab fragments of IgG were generated from antibodies raised against each of these synthetic peptides. These synthetic peptides and Fab fragments were used in the present study to identify the region of the PH-20 protein that is involved in intracellular signaling.

Results from this study demonstrate an HA binding domain (amino acids 205–225) of macaque sperm PH-20 that is involved in cell signaling events (i.e., increase in $[Ca^{2+}]_i$) and that is separate from the active sites responsible for hyaluronidase activity. Here, we demonstrate that HA binds to PH-20 on the plasma membrane of living sperm and in sperm extracts as measured with a microplate assay. It was necessary to use OG extracts of whole sperm for the microplate assay in order to have a large enough quantity of PH-20 available for each experiment. As a consequence, these extracts included PH-20 from the inner acrosomal membrane in addition to plasma membrane PH-20. The Fab fragments of anti-Peptide 2 IgG bind to the inner acrosomal membrane of macaque sperm as shown by immunogold labeling (data not shown). The function of inner acrosomal membrane PH-20 is unclear and there is no evidence that it is involved in cell signaling, but the present study suggests that it is capable of binding HA through the Peptide 2 domain.

In a previous study, we showed that Fab fragments of IgG from antibodies to Peptides 1 and 3 were capable of inhibiting hyaluronidase activity of PH-20 extracted from the plasma membrane or from whole sperm (Yudin et al., 2001). In the present experiments, quercetin was included in the microplate assay to reduce the binding of HA to the active sites of hyaluronidase (Li et al., 1997b). Under these conditions, Fab fragments from anti-Peptide 2 IgG but not from anti-Peptide 1 or anti-Peptide 3 significantly reduced HA binding to sperm extracts. These data indicate that there is an HA binding domain of PH-20 which is separate from the hyaluronidase catalytic domains. The conclusion that the HA binding domain is in the Peptide 2 region is supported by the fact that the only synthetic peptide that bound HA was Peptide 2, and a recombinant protein containing the Peptide 2 domain also bound HA. The absence of HA binding sites in the C-terminal region of PH-20 is suggested by the lack of HA binding to Peptide 4 and to the recombinant protein E12 that contains the C-terminal domains. Since the Fab fragments of anti-Peptide 2 IgG were the only anti-peptide Fabs that inhibited increases in $[Ca^{2+}]_i$, we also conclude that the Peptide 2 domain is involved in the HA-induced signaling in sperm that is mediated by plasma membrane PH-20.

The Peptide 2 region of the PH-20 molecule contains a cluster of basic amino acids with neutral amino acids in between (BxxBxxB), an arrangement that is similar to the RHAMM HA binding motif (B(X7)B) (Yang et al., 1994). As shown in Table 1, this region is highly conserved in hyaluronidases and is similar to the heparin binding motif (BxxBxBxxB).(Ferran et al., 1992) The presence of an HA binding motif in this region also is supported by binding of HA to the recombinant protein G3 (amino acids 143–510) which encompasses the Peptide 2 region, but not to the recombinant protein E12 (amino acids 291–510), which lacks the Peptide 2 region. Arming et al. (1997) substituted the highly conserved terminal arginine within this motif and found that it caused a substantial loss of hyaluronidase activity, which would suggest that HA binding in this region also is necessary for hyaluronidase activity. However, in contrast to the inhibition of macaque sperm hyaluronidase activity by Fab fragments of IgG from anti-Peptides 1 and 3 (Yudin et al., 2001), Fab fragments of anti-Peptide 2 IgG did not inhibit macaque sperm hyaluronidase activity (unpublished data).

HABPs (hyaladherins), comprise a large family of proteins including the link proteins which interact with HA to form complexes that stabilize the ECMs of connective tissues in cartilage, skin and brain

(reviewed by Toole, 1990; Day, 1999). Other link proteins, such as CD44, TSG-6, RHAMM, and ICAM are cell surface receptors that interact with HA to facilitate changes in cell behavior (Chen and Abatangelo, 1999). These HA-induced responses include cell migration, cell adhesion, embryogenesis, endocytosis of HA, cancer progression, inflammatory response, and mobilization and trafficking of hematopoeitic cells (Toole, 1991; Neame and Barry, 1993; Kohda et al., 1996; Delpech et al., 1997; Borland et al., 1998; Day, 1999; McCourt, 1999). None of the previously described cell surface HABPs have hyaluronidase activity and all possess transmembrane and cytoplasmic tails. Because it is a GPI-anchored hyaluronidase, PH-20 appears to be a unique cell surface HABP.

In general, HABPs such as CD44 and TSG-6 exhibit some, but not a high degree of amino acid homology within the link module (Bajorath, 2000). The HA binding domain in TSG-6 and CD44 are within the single link module and are composed of tyrosine residues associated with several positively charged amino acids (Bajorath et al., 1998; Day, 1999). The fact that all of the cysteines are conserved within the TSG-6 and CD44 link modules also point to the importance of the secondary structure (Bajorath, 2000). HABPs such as RHAMM lack the link module but contain a B(X7)B motif (Yang et al., 1994) and the degree of HA binding by these proteins is influenced by the presence of additional basic amino acids within the (X7) region (Entwistle et al., 1996). Although PH-20 does not have amino acid sequence homology with other HABPs, the Peptide 2 domain does have tyrosine residues and a significant charge homology with HABPs. This region is highly conserved in sperm hyaluronidases and similar to heparin binding proteins (Table 1).

The interaction of HA with cell surface receptors is known to result in signal transduction events regulating the functions of cells (Bourguignon et al., 1993; Hall et al., 1994; Toole, 1997). For example, many laboratories have demonstrated changes in protein phosphorylation patterns after cells bind HA (Hall et al., 1994, 1996; Ilangumaran et al., 1999; Oliferenko et al., 2000). In fibroblasts this HA-induced tyrosine phosphorylation is associated with cell motility (Yang et al., 1994), and in lymphocytes it is associated with an increase in $[Ca^{2+}]_i$ (Ilangumaran et al., 1999). In mammalian sperm, HA has been shown to bind to HABPs (Kornovski et al., 1994; Ranganathan et al., 1994), and one of these HABPs has been identified as a tyrosine kinase on the flagellum that is phosphorylated only in motile sperm (Ranganathan et al., 1994). However, in macaque sperm we can demonstrate FITC-HA binding only to the sperm head, and PH-20 is the only macaque sperm protein to which biotinylated-HA binds on blots. (Cherr et al., 1999).

Previous studies from our laboratory have demonstrated that $[Ca^{2+}]_i$ increases when sperm are exposed to HA (Sabeur et al., 1998; Cherr et al., 1999), and that aggregation of plasma membrane PH-20 by anti-PH-20 IgG (but not Fab) results in a similar increase in $[Ca^{2+}]_i$ (Yudin et al., 1998; Cherr et al., 1999). Macromolecular HA aggregates over the head of the sperm (Cherr et al., 1999), and it is likely that aggregation of the GPI-anchored PH-20 that binds this HA is responsible for subsequent increases in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ increase due to HA exposure in human and macaque sperm is not sufficient to induce the acrosome reaction (Yudin et al., 1998; Cherr et al., 1999), but does dramatically increase the number of sperm that undergo the acrosome reaction in response to zona pellucida (Vandevoort et al., 1997; Sabeur et al., 1998; Tollner et al., 2000). Signal transduction events including tyrosine phosphorylation and increased $[Ca^{2+}]_i$ have been shown to follow aggregation of GPI-anchored, surface proteins in other cell types (reviewed by Ferguson, 1992; Harder and Simons, 1999). We hypothesize that the signaling function of PH-20 results from its association with an unidentified transmembrane protein. In our model, the binding of HA to the Peptide 2 domain induces aggregation of PH-20 along with the associated transmembrane protein, and as a result, $[Ca^{2+}]_i$ increases and acrosomal swelling is initiated (Yudin et al., 1998; Cherr et al., 1999). These events, which take place as sperm traverse the cumulus investment, increase the efficiency of acrosome reactions after sperm bind to the zona pellucida (Vandevoort et al., 1997).

In summary, the present study has demonstrated that the Peptide 2 region (amino acids 205–235) of the PH-20 molecule is an HA binding site. This domain shares amino acid sequence homology with PH-20 proteins from different species, as well as charge homology with other HABPs. Two domains of PH-20 have been identified as active sites for PH-20 hyaluronidase activity (Yudin et al., 2001). However, data from this study suggest that these sites are not significant HA binding domains. This new information suggests a basis for two distinct functions of sperm plasma membrane PH-20, an HA binding function associated with intracellular signal transduction, and an enzymatic function associated with degradation of the cumulus ECM.

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