Characterization and Molecular Cloning of a Putative Binding Protein for Heparin-binding Growth Factors*

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A novel M, 17,000 heparin-binding protein was purified from culture medium conditioned by A431 human epidermoid carcinoma cells. This protein, designated HBp17, was found to bind the heparin-binding peptide growth factors HBGF-1 and HBGF-2 in a non-covalent, reversible manner. In addition HBp17 wasfound to inhibit the biological activities of both HBGF-1 and HBGF-2. Both the binding and inactivation of HBGF-1 and HBGF-2 by HBp17 were abolished by heparin. Full-length 1163-base pair HBp17 cDNA was cloned and sequenced by using the polymerase chain reaction technique. The deduced primary structure of HBp17 consisted of 234 amino acids including each of five partial peptide sequences obtained from proteolytic fragments of purified HBp17. The encoded protein included a 33-residue N-terminal signal sequence for secretion and a single potential N-linked glycosylation site. No homology with any known protein was found for the deduced primary structure of HBp17. The expression of HBp17 mRNA was found to occur preferentially in normal human keratinocytes and in squamous cell carcinomas. This pattern of HBp17 gene expression suggests that this binding protein for HBGFs 1 and 2 has a physiological role in squamous epithelia.

Heparin-binding growth factors (HBGF) are a family of seven polypeptide growth factors that include HBGF-1 (acidic FGF), HBGF-2 (basic FGF), K-FGF, Int-2, FGF-5, KGF, and FGF-7 (1-3). Of these HBGF-1 and HBGF-2 have been implicated in a number of biological processes in vitro and in vivo such as growth stimulation of mesodermal- and neuroectodermal-derived cells, angiogenesis, wound healing, and tissue regeneration (1, 3, 4). Both HBGF-1 and HBGF-2 were initially isolated from neural tissue as M, 16,000-18,000 polypeptides (5, 6). They are 55% identical in amino acid sequence (7), they have similar biological activities (1, 3), and they may act through the same cell surface receptor molecules (8). However, HBGF-1 and HBGF-2 have several functionally defined differences that are based on their interactions with heparin. HBGF-2 is biologically more potent than HBGF-1 in the absence of heparin (9); the activity of HBGF-1 but not HBGF-2 is potentiated by heparin (10, 11); and HBGF-2 has a greater affinity for immobilized heparin than HBGF-1 (12).

Tumor angiogenesis is a potential rate-limiting step in tumor development and metastasis (13-15). Both HBGF-1 and HBGF-2 have been shown to have strong angiogenic activity (16-18). Since many tumors have been found to produce HBGF-1 or HBGF-2 (1, 3), they may be directly involved in the vascularization of tumors (14). However, neither HBGF-1 nor HBGF-2 has conventional signal sequences for secretion (19, 20), and many cells that produce HBGF-1 or HBGF-2 release them at very low levels (21-23). In addition HBGFs have been found to associate with extracellular matrix components (23-25), but the mechanisms by which HBGFs 1 and 2 are translocated to extracellular spaces remain unknown.

Binding proteins for growth factors including epidermal growth factor (EGF) (26, 27), nerve growth factor (NGF) (28, 29), insulin-like growth factors (IGFs) (30-33), insulin (34), platelet-derived growth factor (PDGF) (35), and transforming growth factor-β (TGF-β) (36-38) have been identified, and binding proteins have been suspected to be involved in making HBGF-1 and HBGF-2 available to cell surface receptors (1). Dennis et al. (39) have found that α2-macroglobulin is a binding protein for HBGF-2, but because it appears to bind HBGF-2 irreversibly, α2-macroglobulin is not likely to function as a physiological carrier of HBGF-2. Here we describe a heparin-binding protein purified from culture medium conditioned by A431 human epidermoid carcinoma cells (40, 41), which was observed associated with an HBGF-2 activity produced by these cells. This protein, designated HBp17, is shown to bind to both HBGF-1 and HBGF-2 in a reversible manner and thus may act as a carrier for these HBGFs. The effects of HBp17 on the biological activities of HBGF-1 and HBGF-2 are examined, and the sequence of cloned HBp17 cDNA is reported.

MATERIALS AND METHODS

Cell Culture and Cell Proliferation Assay—The epidermoid carcinoma cells A431-AJC, a clone of A431 cells (40, 41), and Kano's were cultured in DME/F-12 nutrient medium. A431-4 (42), a nonmutogenic clonal variant of A431 cells, was obtained from Dr. T. Kawanoto (Biochemistry Dept., Okayama University Dental School, Okayama, Japan) and was cultured in DME/F-12 medium supplemented with 10% fetal bovine serum.

1 T. Okamoto, unpublished results.
adenocarcinomas, DU145 prostate adenocarcinoma (49), and SKCO-cervical adenocarcinoma (46), MCF-7 (47) and BT-20 (48) mammary cinomas, SK-MEL-1 melanoma (45), SK-LMS-1 sarcoma (45), HeLa cultured in DME/F-12 medium with 2% FBS. Normal human keratocytes were purchased from Clonetics Corp. (San Diego, CA) and cultured in KGM medium from the same supplier. Swiss 3T3 and human foreskin fibroblasts were cultured in DME/F-12 medium with 10% FBS.

Cell proliferation was assessed by the incorporation of [3H]thymidine into Swiss 3T3 cells. 1 x 10^4 cells/well were grown to confluence in DME/F-12 medium with 10% FBS. The cells were serum starved in nutrient medium for 24 h, and growth factor—conditioned by A431-AJC cells were concentrated 10-fold with a Sephadex G-50 column (Pharmacia LKB Biotechnology Inc.) at 37°C in 24-well plates in DME/F-12 medium with 10% FBS. The concentration of the HBGFs and the binding of HBGF-1 to Hep2 human hepatoma cells (54) were determined as described by Kopp et al. (55).

Radiolabeled HBGF-1 and HBGF-2 were covalently cross-linked to HBGF-1 with disuccinimidyl suberate (DSS) (Pierce Chemical Co.). 4 µg of HBGF-1 were incubated with 1 x 10^6 cpm [35S]HBGF-1 in a total volume of 20 µl at 22°C for 2 h. DSS was added to a final concentration of 1 mM, and the reaction was incubated at 4°C for 20 min. The DSS sample buffer was added, the samples were cooled, and the cross-linked complexes were electrophoresed in a 15% polyacrylamide gel by the method of Laemmli (50).

HBGF-binding Protein—Twenty liters of DME/F-12 medium conditioned by A431-AJC cells were concentrated 10-fold with a hollow fiber concentrator (Amicon, Danvers, MA) and passed over a heparin-Sepharose column (Pharmacia LKB Biotechnology Inc.) as described by Wu et al. (52). Four of the resulting peptides were used as a test standard the sequenator gave initial yields of 40% and repetitive yields in excess of 90%.

The amino acid sequences EQKDTLGN and GNTQIKQK from the N terminus of HBGF-1 and CFVAGNPT from peptide P2 were used to synthesize 23-residue degenerate oligonucleotide primers with a model 381A DNA synthesizer (Applied Biosystems). The primer pools were: A, 5'GACGXXAGXXGAYCNYNNGNNA-3'; B, 5'-GNGNAAYCNAXATAXAAXCAAXA-3'; and C, 5'-ACXXCAANAXCGCNCNTXGGNTG-5'. X was A or G, Y was T or C, X was A, T, or C, and N was any nucleotide.

Identification of HBGFs, Receptor Binding Assay, and Affinity Cross-linking to Receptor—Bovine HBGF-1 (Upstate Biotechnology, Inc., Lake Placid, NY) and HBGF-2 (R and D Systems) were labeled with Na[35S]I (Amersham Corp.) to specific activities of 5.9 x 10^6 and 2 x 10^6 cpm/mg, respectively. The iodination of the HBGFs and the binding of HBGF-1 to Hep2 human hepatoma cells (54) were determined as described by Kopp et al. (55).

DNA Amplification by Polymerase Chain Reaction (PCR)—cDNA sequences were amplified by PCR as described by Saiki et al. (56). First strand cDNA was synthesized from 1 µg of poly(A)+ mRNA isolated from HEPI cell line (Invitrogen, San Diego, CA). PCR was done with a GeneAmp kit (Perkin Elmer-Cetus) using the synthetic oligonucleotide primers described above. 40 cycles of amplification were carried out with the following denaturation, annealing, and extension steps: 94°C x 1 min; 40°C x 2 min, and 72°C x 3 min for reactions with degenerate primers or 94°C x 40 s; 55°C x 1 min, and 72°C x 2 min for RACE reactions. The final amplification cycle was followed by a 10-min extension reaction at 72°C. Rapid amplification of DNA end (RACE) reactions were done as described by Frohman et al. (57).

DNA Cloning and Sequencing—PCR products were purified by electrophoresis in agarose gels and were blunted end-ligated into the SK Bluescript vector (Stratagene, La Jolla, CA). Recombinant SK plasmids were isolated (QIAGEN, Chatsworth, CA) and inserted into the M13mp18 vector (Beverly, MA), and inserts were sequenced by the dyeoxy chain termination method (58) with a Sequenase version 2.0 DNA sequencing kit (U. S. Biochemical Corp.). Complete HBGF cDNA and amino acid sequences were used with the FASTA search program (59) to find homologous sequences in the GenBank (release 67; IntelliGenetics, Inc., Mountain View, CA) and PIR (release 26; National Biomedical Research Foundation (NBRF), Washington, D. C.) data bases.

Sanctuary and Northern Hybridizations—For Southern hybridizations RACE products were separated in 1.2% agarose gels and transferred to nitrocellulose (Micron Separation, Inc., Westboro, MA). The membrane was hybridized with a 32P-labeled 192-bp PCR product in 5 x SSPE (1x = 0.18 M NaCl, 10 mM sodium phosphate, pH 7.4, 1 mM EDTA), 5 x Denhardt's solution (1x = 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), 50% formamide, 0.05% SDS, and 10 µg/ml salmon sperm DNA for 10 h at 42°C. The membrane was washed three times at 42°C with 1 x SSC (0.15 M NaCl, 0.018 M sodium citrate, pH 7.0) containing 0.1% SDS. The dried membrane was exposed to Kodak XAR5 film.

Poly(A)+ RNA extracted from cells with RNAzol (Cinna/Biotex, Friendswood, TX) and isolated by chromatography on oligo(dT) cellulose (Collaborative Research, Bedford, MA) was used in Northern hybridizations. 5 µg of each mRNA were electrophoresed in a 1% agarose gel and transferred to a nylon membrane (Zeta-Probe; Bio-Rad). Hybridization and washing conditions were the same as those for Southern hybridizations. Poly(A)+ RNAs from HepG2 (55), Hep3B (55), SK-Hep-1 (45), and normal fetal liver cells were kindly provided by Dr. W. L. McKeohan (W. Alton Jones Cell Science Center, Lake Placid, NY).
RESULTS

Serum-free culture medium conditioned by A431 cells was subjected to heparin affinity chromatography. Heparin-bound proteins that eluted in 0.65–0.95 M NaCl were associated with a considerable level of mitogenic activity when assayed by [³H]thymidine incorporation into serum-starved Swiss 3T3 cells (results not shown). This mitogenic activity was further purified by gel filtration HPLC and C-4 reverse-phase HPLC (Fig. 1A). In the latter step the mitogenic activity co-eluted with a minor absorbance peak in 31% ACN and was separated from the major absorbance peak, which eluted in 27% ACN. After rechromatography on C-4 the major absorbance peak was resolved as a M,17,000 band by SDS-PAGE in a 15% polyacrylamide gel run under reducing conditions (inset, panel B). Edman degradation of purified HBp17 yielded an N-terminal sequence of KVVSEQKDTLGNTQIKQKSRPGNKGKFVTKDQAN.

FIG. 1. Purification of HBp17 by HPLC. A, following heparin affinity chromatography and gel filtration HPLC the pooled protein from A431-conditioned medium was fractionated by RP-HPLC on an analytical C-4 column that was developed with a linear gradient of 23–33% ACN in 0.1% trifluoroacetic acid in 30 min at a flow rate of 1 ml/min. 1-ml fractions were collected and assayed for mitogenic activity on 3T3 cells as indicated by the hatched bars. B, the major absorbance peak, which eluted from the RP-HPLC column in 27% ACN, was rechromatographed on a C-4 column; the column was developed with a linear gradient of 25–30% ACN in 30 min at a flow rate of 1 ml/min. The purified material was resolved into a major silver stained band of M,17,000 by SDS-PAGE in a 15% polyacrylamide gel run under reducing conditions (inset, panel B). Edman degradation of purified HBp17 yielded an N-terminal sequence of KVVSEQKDTLGNTQIKQKSRPGNKGKFVTKDQAN. C, A431 HBGF-2 (lane A) and bovine brain HBGF-2 (lane B) were electro-phoresed in a 15% polyacrylamide-SDS gel and blotted to nitrocellulose. Membrane-bound HBGF-2 was detected by indirect enzyme-linked immunoassay.
Fig. 2. Interactions between A431 HBGF-2 and HBp17. A, heparin affinity-purified HBGF-2 from A431-conditioned medium was passed over a column of immobilized HBp17, and the column was eluted with 1 mg/ml heparin. Aliquots of the flow-through (○), eluate (□), and nonfractionated sample (●) were normalized and assayed for the stimulation of ["H]thymidine incorporation by Swiss 3T3 cells. B, A431 HBGF-2 was left untreated (○) or it was incubated with (□) or without (△) 0.5 μg/ml HBp17 in 0.1% trifluoroacetic acid (pH 2.0) for 4 h at room temperature. Aliquots were then assayed for mitogenic activity on Swiss 3T3 cells.

In a functional assay purified HBp17 inhibited the abilities of both HBGF-1 and HBGF-2 to stimulate the incorporation of ["H]thymidine into 3T3 cells (Fig. 4, A and B). 1.5 μg/ml HBp17 half-maximally inhibited the mitogenic activity of 10 ng/ml HBGF-1, while 12 μg/ml HBp17 half-maximally inhibited the activity of 0.5 ng/ml HBGF-2. Thus, this effect of HBp17 appeared more potent for HBGF-1 than HBGF-2. By contrast, HBp17 did not inhibit PDGF-stimulated DNA synthesis in 3T3 cells (results not shown). The inhibitory effect of HBp17 on the activities of HBGF-1 and HBGF-2 was not observed in the presence of 10 μg/ml heparin. To determine whether the inhibition of HBGF activity by HBp17 occurred at the level of ligand-receptor binding 125I-HBGF-1 was incubated with HepG2 human hepatoma cells in the presence or absence of HBp17 under conditions in which HBGF-1 was not internalized (55). HBp17 inhibited the binding of radio-labeled HBGF-1 to intact HepG2 cells in a concentration-dependent manner indicating that the inhibition of HBGF-1 activity by HBp17 resulted from decreased ligand binding (Fig. 4C). In the presence of heparin no inhibition of binding could be detected (data not shown).

To determine the entire primary structure of HBp17 the...
PCR method (56) was used to amplify and clone HBp17 cDNA from A431-derived poly(A') mRNA. Four V8 protease-generated fragments of purified HBp17 were purified by RP-HPLC (Fig. 5A) and sequenced in order to design PCR primers. None of the N-terminal peptide sequences determined were homologous to GenBank DNA sequences (release 67) or NBRF-PIR protein sequences (release 26). Three pools (A–C) of 23 base degenerate primers were designed based on the N-terminal sequences of HBp17 and V8 protease-generated fragment P2 (Fig. 5A). The oligonucleotide primer sequences used are described under "Materials and Methods." Polymerase chain reactions with C primers paired with either A or B primers yielded cDNA products that differed in length with the known positions in the N terminus of HBp17 of the peptides corresponding to primer pools A and B. The larger PCR product was cloned in the SK Bluescript vector and sequenced; the insert consisted of 192 bp and contained sequences corresponding to all three primer pools. The 5' and 3' regions flanking this 192-bp cDNA sequence were obtained by using the RACE technique (57). The 3' RACE and 5' RACE reactions yielded 0.95- and 0.35-kb fragments, respectively, which hybridized to the original 192-bp HBp17 cDNA (Fig. 6). These fragments were also cloned in the SK Bluescript vector and sequenced.

The full-length sequences of HBp17 cDNA and its deduced protein product are depicted in Fig. 7. HBp17 cDNA consisted of 1146 bp excluding the poly(A) tail, and its longest open reading frame encoded 234 amino acids, which included the five amino acid sequences obtained from the N termini of HBp17 and V8 protease-generated peptides P1 to P4. The first methionine codon of this open reading frame started at nucleotide 98 and was immediately preceded by the sequence 5'-GCTGCAGCC-3', which is similar to the consensus translation start sequence 5'-GGCGCCPuCC-3' of vertebrate mRNAs (64). Thus, this methionine codon most likely initiated the translation of HBp17. In addition there was an in-frame stop codon 90 nucleotides upstream of this methionine codon. The amino acid sequence from residue 6 to 21 following the initiation methionine was very hydrophobic and could form the core of a signal sequence for secretion. Compared with other signal peptide sequences (65, 66), the most probable cleavage site for the HBp17 signal sequence was Ser-33. This prediction was in agreement with the observed N-terminal residue of purified HBp17 being Lys-34. There were two consecutive stop codons at HBp17 cDNA nucleotides 800 and 803, which were followed by at least 341 bp of 3' non-coding region. A single poly(A) addition signal sequence 5'-AA-TAAA-3' (67) was located at nucleotide 1125. From this analysis we deduced that mature HBp17 was a secreted protein, which was derived from the proteolytic processing of a 234-residue precursor molecule. The HBp17 precursor had a single potential N-linked glycosylation site at Asp-99. Homology searches of the NBRF-PIR and GenBank data bases revealed no sequences homologous to HBp17 or HBp17 cDNA, respectively.

Northern hybridizations with the 192-bp HBp17 cDNA fragment as a radiolabeled probe were used to examine a number of normal and tumor-derived human cells for the presence of HBp17 mRNA (Fig. 8). A single 1.2-kb mRNA species was detected in A431 poly(A) RNA, which was consistent with the deduced size of full-length HBp17 cDNA (Fig. 7). This 1.2-kb HBp17 mRNA was also expressed in normal keratinocytes and in the five squamous cell carcinomas ME180, FaDu, A253, Kane, and SCC-25 in addition to A431 cells (Fig. 8). HBp17 mRNA was not detected in normal human foreskin fibroblasts, fetal liver cells, HepG2 and SK-HEP-1 hepatoma cells, or MCF-7 mammary adenocarcinoma cells (Fig. 8). Also negative were SK-LMS-1 sarcoma cells, SK-MEL-1 melanoma cells, Hep3B hepatoma cells, and the following adenocarcinoma cell lines: HeLa, DU 145, BT-20, and HT-29 (data not shown). SKCO-1 colon carcinoma was the only adenocarcinoma tested in which HBp17 mRNA could be detected. Interestingly, the A431-4 nontumorigenic variant of A431 cells (42) did not express detectable levels of HBp17 mRNA (Fig. 8).
Indeed, as HBGF-2 binds to a form of α2-macroglobulin that is rapidly cleared from the circulation, it is likely that α2-macroglobulin would mediate the inactivation and elimination of free HBGF-2 released into the plasma (38, 69). A similar role has been proposed for the binding of α2-macroglobulin to PDGF (35) and TGF-β (36, 37).

In this study we have isolated from serum-free culture medium conditioned by A431 human epidermoid carcinoma cells a 17,000 heparin-binding protein, designated HBpl7, that had the attributes of a binding protein for HBGFs 1 and 2. Purified HBpl7 bound HBGF-2 (Fig. 2) and HBGF-1 (Fig. 3) in a noncovalent manner. These interactions could be reversed by either free HBpl7 or heparin, which suggested that the binding of HBpl7 and heparin to HBGFs 1 and 2 were antagonistic events. In addition HBpl7 inhibited the biological activities of HBGFs 1 and 2, as measured by the stimulation of [3H]thymidine incorporation by Swiss 3T3 cells (Fig. 4), through the inhibition of receptor-mediated ligand binding. The inhibition of HBGF activity by HBpl7 could also be blocked by heparin. These results might be expected if HBpl7 and heparin were to bind to overlapping regions of HBGFs 1 and 2, and they suggest a mechanism mediated by heparin-like molecules by which HBGFs 1 and 2 could be liberated from complexes with HBpl7 after being delivered to their sites of action. The interactions between HBpl7 and HBGFs 1 and 2 were specific in that HBpl7 did not inhibit PDGF-stimulated [3H]thymidine incorporation by Swiss 3T3 cells (Fig. 4), and its sequence was determined. The cloned HBpl7 cDNA consisted of a total of 1146 base pairs followed by a poly(A) tract (Fig. 7). A 192-bp cDNA fragment hybridized to a single 1.2-kb species of A431 poly(A+) RNA (Fig. 8), which suggested that the complete sequence of HBpl7 mRNA had been obtained. The cloned HBpl7 cDNA consisted of a total of 1146 base pairs followed by a poly(A) tract (Fig. 7). A 192-bp cDNA fragment hybridized to a single 1.2-kb species of A431 poly(A+) RNA (Fig. 8), which suggested that the complete sequence of HBpl7 mRNA had been obtained.

**DISCUSSION**

Binding proteins distinct from cellular receptor molecules have been identified in tissues or plasma for peptide hormones or growth factors including epidermal growth factor (26, 27), transforming growth factor-β (36-38), and HBGF-2 (basic FGF) (39). In general the plasma-derived binding proteins served to inactivate their ligands and thus regulate ligand activity by reducing the concentration of free ligand. α2-Macroglobulin, a M, 720,000 serum protease inhibitor (68), has been found to act as a binding protein for PDGF (35), TGF-β (36, 37), and HBGF-2 (39). Since this serum glycoprotein binds HBGF-2 in an irreversible manner, it does not appear to function as a carrier protein for HBGF-2 (39).

**FIG. 7. HBpl7 cDNA and the deduced amino acid sequence of HBpl7.** The nucleotide sequence of PCR-derived HBpl7 cDNA is presented with the deduced primary structure of HBpl7. Amino acid sequences determined from intact HBpl7 and four of its V8 protease-generated peptides are underlined. The sole potential N-linked glycosylation site at residue 99 is also underlined. Nucleotide sequences obtained from the N terminus of HBpl7 and four of its sequence has been assigned GenBank accession number M60047.

**FIG. 8. Northern analysis of HBpl7 mRNA expression.** RNAs from the following sources were examined for the expression of HBpl7 mRNA: A431, MCF-7, ME180, A253, FaDu, A431 and SCC-25 squamous cell carcinomas; HepG2 and SK-Hep-1 hepatoma cell lines; MCF-7 mammary adenocarcinoma; SK-O-1 colon adenocarcinoma; human foreskin fibroblasts (HFF); and normal human keratinocytes (HKC). 5 µg of poly(A) RNA from each source were electrophoresed in a 1% agarose gel and blotted to a nylon membrane. The membrane was probed with a 192-bp [32P]HBpl7 cDNA fragment.
frame terminated with two consecutive stop codons. The deduced primary translation product of HBp17 mRNA was a 26,247 precursor molecule with a 33-residue N-terminal signal peptide of M, 3594 and a single potential N-linked glycosylation site at residue 99. The detection of Asn-99 during the sequencing of HBp17 peptide P2 (Fig. 7) suggested that this residue was not glycosylated. The HBp17 precursor signal peptide predicted by comparison with known signal sequences (65, 66) coincided with that determined by N-terminal sequence analysis of the mature protein. Nucleotide or amino acid sequences homologous to HBp17 mRNA and pre-HBp17 could not be found in the GenBank (release 67) or NBRF-PIR (release 26) databases, respectively, which indicated that HBp17 was a novel protein.

The molecular weight of mature HBp17 estimated from its mobility in SDS-PAGE was significantly smaller than the M, 22,653 determined from its deduced amino acid sequence. This discrepancy suggested either that HBp17 assumed a highly compact conformation or that HBp17 was proteolytically processed at its C-terminal end. By amino acid analysis, purified HBp17 contained no methionine residues (data not shown), which suggested that HBp17 isolated from A431-conditioned medium was truncated prior to Met-168 of the deduced HBp17 precursor molecule (Fig. 7). Thus, the precise C-terminus of mature HBp17 is not known. As mature HBp17 contained neither hydrophobic sequences of sufficient length for a transmembrane domain nor N-linked sequences for a signal peptide predicted by comparison with known signal sequences, it is not known whether HBp17 is a membrane protein. The Precise C terminus of mature HBp17 is not known. As mature HBp17 contains neither hydrophobic sequences of sufficient length for a transmembrane domain nor N-linked sequences for a signal peptide predicted by comparison with known signal sequences, it is not known whether HBp17 is a membrane protein.

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REFERENCES


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HBGF-binding Protein