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Molecular Cloning of Human Fibroblast Hyaluronic Acid-binding Protein Confirms Its Identity with P-32, a Protein Co-purified with Splicing Factor SF2

HYALURONIC ACID-BINDING PROTEIN AS P-32 PROTEIN, CO-PURIFIED WITH SPLICING FACTOR SF2*

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The purification of a 68-kDa hyaluronic acid-binding protein (HA-binding protein), a homodimer of 34 kDa that binds specifically to hyaluronic acid, has been reported earlier by us (Gupta, S., Batchu, R. B., and Datta, K. (1991) *Eur. J. Cell Biol.* 56, 58–67). Here, we report the isolation of a partial cDNA clone from a λ gt_{11} cDNA expression library of human skin fibroblast by immunoscreening with HA-binding protein antiserum. The internal polypeptide sequence (83 residues) of the purified hyaluronic acid-binding protein is identical to the predicted protein sequence derived from hyaluronic acid-binding protein cDNA, suggesting the authenticity of the clone. Interestingly, this hyaluronic acid-binding protein cDNA sequence has complete homology with the cDNA sequence of a protein P-32, co-purified with the human pre-mRNA splicing factor SF2 (Krainer, A. R., Mayeda, A., Kozak, D., and Binns, G. (1991) *Cell* 66, 383–394). Furthermore, the data on the N-terminal sequence of hyaluronic acid-binding protein and the predicted polypeptide of P-32 revealed the identical coding sequence of 209 amino acids for both the proteins. As the identity and functional characterization of P-32 have not yet been reported, P-32 cDNA was expressed in *Escherichia coli*, and the recombinant P-32 protein was purified by hyaluronic acid-Sepharose affinity chromatography. The recombinant P-32 protein showed immunocross-reactivity with the polyclonal antibodies raised against HA-binding protein. The predicted amino acid sequence of the protein fulfilled the minimal criteria for binding to hyaluronic acid, *i.e.* two basic amino acids flanking a seven-amino acid stretch, as reported for other hyaluronic acid-binding proteins. Furthermore, the hyaluronic acid affinity of the recombinant P-32 protein was confirmed by biotinylated hyaluronic acid binding assay. The binding of recombinant P-32 protein to biotinylated hyaluronic acid can be competed only with excess unlabeled hyaluronic acid, confirming its specificity toward hyaluronic acid. All these results suggest that both P-32, co-purified with the human pre-mRNA splicing factor SF2, and 34-kDa hyaluronic acid-binding protein reported by us are the same protein and that it is a new member of the hyaluronic acid-binding protein family, the "hyaladherins."

Hyaluronic acid (HA),[1] a viscoelastic, high molecular weight, nonsulfated glycosaminoglycan, is ubiquitously present in both the extracellular space and the nucleus of higher animals (1–5). The biological functions of HA include not only physiological roles such as maintenance of matrix structure and water balance (6, 7) but also cellular functions like proliferation (6, 8), tissue recognition (7), and locomotion (9). The cell type-specific functions of HA are mediated through its interaction with HA-binding proteins (7).

A number of extracellular as well as cell-surface HA-binding proteins are known to mediate the multifaceted regulations of HA-induced cellular functions (10–19). Due to the presence of defined HA-binding motif, these proteins are expected to belong to one family, recently, referred to as "hyaladherins" (20). Few of them are molecularly characterized, and their sequence data confirm their distinction.

The purification of 68-kDa homodimer (34-kDa subunit) HA-binding protein from rat kidney tissue and its specificity toward hyaluronic acid were confirmed earlier from our laboratory (21). The differential expression of this glycoprotein on the cell surface of AK-5, a transplantable histiocytic tumor cell line, and its role in cell adhesion were also demonstrated (22). Ranganathan *et al.* (23) observed the *in vivo* phosphorylation of this protein in motile spermatozoa and established its role in sperm maturation, motility, and fertilization. To understand the regulation of expression of this protein and its precise role in cellular functions, we have cloned the gene encoding this 34-kDa HA-binding protein. A λ gt_{11} human skin fibroblast cDNA expression library was screened with antibodies raised against this HA-binding protein. Partial cDNA sequence analysis and the deduced amino acid sequence match with the amino acid sequence of purified HA-binding protein. Interestingly, the sequence analysis of the HA-binding protein confirms its homology with P-32, a protein co-purified with pre-mRNA splicing factor SF2 from human HeLa cells described by Krainer *et al.* (24, 25). The identity and functional characterization of P-32 protein was not known earlier. Here, we report the 34-kDa HA-binding protein to be the same as P-32 from sequence analysis and also describe the purification of recombinant P-32 protein by HA-Sepharose column, thus again confirming the identity of P-32 protein as an HA-binding protein. This result is also supported by immunological and biochemical studies showing the specificity of HA affinity of this protein.

EXPERIMENTAL PROCEDURES

Materials—Hyaluronic acid (human umbilical cord, grade 1) was purchased from Sigma. AH-Sepharose 4B was from Pharmacia Biotech

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[1] The abbreviations used are: HA, hyaluronic acid; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-1-thio-β-D-galactopyranoside; kb, kilobase; RNP, ribonucleoprotein.

Inc. Human skin fibroblast λ gt_{11} cDNA libray was from Clontech, Palo Alto, CA. CircumVent™ thermal sequencing kit was procured from New England Biolabs. Enhanced chemiluminescence (ECL) Western blot kit was from Amersham Corp. All other routine reagents were purchased from Sigma.

Purification of HA-binding Protein and Development of Polyclonal Antibodies—HA-binding protein has been purified from rat kidney tissue by using HA-Sepharose affinity chromatography (21). Briefly, HA from human umbilical cord (grade 1) was coupled to AH-Sepharose 4B (Pharmacia) according to the manufacturer's instructions, resulting in an HA-Sepharose gel. The supernatant after centrifuging the homogenate at 48,000 × *g* was fractionated by 70% ammonium sulfate at 4 °C for 30 min, dialyzed against phosphate-buffered saline and loaded onto the HA-Sepharose column. Nonspecifically bound proteins were washed out with 10 volumes of 0.5 M NaCl in 10 mM phosphate buffer, pH 7.2, and the HA-binding protein was eluted with 0.2 M glycine-HCl, pH 2.2, at a flow rate of 20 ml/h. Polyclonal antibodies against this purified HA-binding protein were developed in rabbit. IgG fraction was purified by protein A-Sepharose CL-4B column and used for immunoscreening a human skin fibroblast λ gt_{11} cDNA expression library.

Partial Amino Acid Sequence Analysis and Determination of N Terminus of HA-binding Protein—The purified HA-binding protein was denaturated in 6 M guanidine overnight and precipitated with 10 volumes of ethanol. The precipitate was suspended in 0.2 M ammonium hydrogen carbonate and cleaved with endoproteinase Asp-N (enzyme: substrate ratio, 1:50) at 23 °C overnight. The mixture was acidified to pH 1 with trifluoroacetic acid, and the peptides were separated on a C-18 reversed phase column (Vydac, 4.6 × 250 mm) using 0.1% trifluoroacetic acid (solvent A) and 70% acetonitrile in 0.1% trifluoroacetic acid (solvent B) with a gradient from 0 to 60% B for 160 min at a flow rate of 0.25 ml/min. Amino acid sequences were determined with gas-liquid phase sequencers 470 A and 473 A (Applied Biosystems) as per the manufacturer's instructions.

Immunoscreening, Cloning, and Sequencing of Human Skin Fibroblast cDNA of HA-binding Protein—A human skin fibroblast λ gt_{11} cDNA library (1.1×10^6 clones; Clontech) was screened with HA-binding protein polyclonal antibodies (IgG) according to the manufacturer's kit protocol. After three rounds of screening, positive signals were chosen. λ DNA was purified by the plate lysate method (26). The cDNA insert was polymerase chain reaction (PCR)-amplified using λ gt_{11} forward and reverse primers and AmpliTaq (Perkin-Elmer) as described in Perkin-Elmer protocol for DNA amplifications. The reaction was carried out at 94 °C (1 min), 55 °C (1 min), and 70 °C (2 min) for a total of 30 cycles following a final 10-min elongation step at 72 °C (27). The PCR product (~0.9 kb) was directly sequenced by Sanger's dideoxynucleotide chain termination method (28) using CircumVent™ thermal cycle sequencing kit (New England Biolabs) and λ gt_{11} forward and reverse primers. A sequence of 400 bases was obtained, and this sequence completely matched with the P-32 cDNA sequence (24) upon alignment. The PCR product was subcloned in *Eco*RV opened pBluescript KS(+) (Stratagene, La Jolla, CA) as described by Sambrook *et al.* (26). The remaining part of cDNA insert in the minipreparation of pBluescript KS(+) was sequenced using two internal primers 5′-TTG GAC AAG AAG ACG AGG CTG-3′ and 5′-AGT TTC ATG GCA GGC TTT GGC-3′ (National Biosciences, Plymouth, MN), which were designed based on the published cDNA sequence of P-32 (24).

Purification of Recombinant P-32 Protein by HA-Sepharose Chromatography—The expression clone representing the mature P-32 protein (209 amino acids) was a generous gift from Dr. Adrian R. Krainer (Cold Spring Harbor Laboratory, NY) and expressed in BL 21(3E) as described (24). Bacterial cultures were induced for 3 h with 0.4 mM IPTG when the A_{600} reached 1. The cells were harvested, washed, and suspended in 0.1 M NaCl, 10 mM Tris-Cl (pH 8.0) and lysed with lysozyme for 30 min at room temperature. Complete lysis was achieved by sonication on ice in 10-s bursts at 30-s intervals. The lysate was centrifuged at 12,000 rpm for 30 min, and clear supernatant containing soluble recombinant P-32 was loaded onto an HA-Sepharose affinity column. Recombinant P-32 was purified using the same protocol used for the purification of tissue HA-binding protein as described before. Polyclonal antibodies against purified recombinant P-32 were raised in rabbit.

SDS-PAGE and Two-dimensional Gel Electrophoresis—SDS-PAGE of purified HA-binding protein, recombinant P-32, and bacterial lysates were performed as described by Laemmli (29). Proteins were stained with 0.1% Coomassie Brilliant Blue in 25% methanol and 10% acetic acid. Two-dimensional gel electrophoresis was done according to the described method (30). Proteins were estimated by the folin phenol method of Lowry *et al.* (31).

Electroblotting and Immunodetection—Proteins were separated on 12.5% SDS-PAGE and transblotted onto a nitrocellulose membrane in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3, as described by Towbin *et al.* (32). Proteins were immunodetected using rabbit anti-HA-binding protein antibodies (1:500) and visualized by nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate detection system (Promega, Madison) using goat anti-rabbit alkaline phosphatase conjugate as secondary antibodies (1:7500).

For reprobing experiment, proteins were transblotted on ECL Hybond nitrocellulose as described before. Proteins were probed first with antirecombinant P-32 protein antibodies (1:500), second with goat anti-rabbit horseradish peroxidase (1:10,000), and bands were visualized by ECL kit by exactly following the manufacturer's instructions (Amersham). After band development, the blot was incubated in 62.5 mM Tris-Cl, pH 6.7 containing 100 mM β-mercaptoethanol and 2% SDS at 50 °C for 30 min for stripping off the bound antibodies and again blocked in 10 mM Tris-Cl, pH 8.0, containing 150 mM NaCl (TBS), 0.05% Tween-20 (TBST), and 5% skimmed milk powder (TBSTS), reprobed with rabbit anti-HA-binding protein antibodies (1:1000), and then processed by following the previous ECL detection method described in the manufacturer's kit.

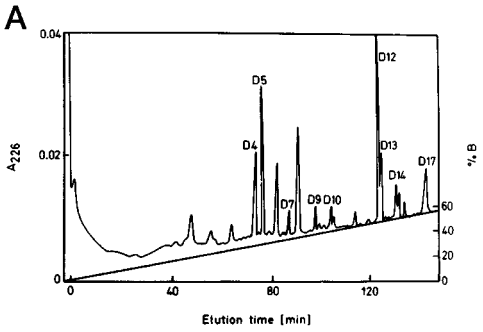
Dot Blot and Transblot Assay Using Biotinylated HA—Biotinylated HA (0.7 mg/ml) was a kind gift from Dr. K. Hoare (University of Texas Medical Branch). Biotinylated HA assay was done according to Yang *et al.* (33). The HA (human umbilical cord) and calf thymus DNA were a commercial preparation, and rat liver total RNA was purified according to Chomczynski and Sacchi (34). Recombinant P-32 protein was dot-blotted on ECL Hybond nitrocellulose membrane in different concentrations in four slots, each having the same amount. Additional protein binding sites were blocked in TBS, TBST, and TBSTS for 2 h. The four blocked membranes containing the same proteins in equal amounts were incubated separately with biotinylated HA (1:4000) alone and in the presence of a 20-fold excess of HA, DNA, or RNA. The unlabeled excess HA, DNA, and RNA were preincubated with biotinylated HA at 37 °C for half an hour before addition. The membranes were washed with TBST for 4 × 10 min and then jointly incubated with avidin peroxidase (Sigma) in 1:1500 dilution in TBST containing 2% milk powder for 1 h. After washing with TBST (4 × 10 min), the membranes were rinsed with TBS and developed simultaneously to visualize the bands with ECL detection method.

For biotinylated HA transblot experiment, exactly the same procedure was followed except that the boiled purified recombinant P-32 protein (with or without 5% β-mercaptoethanol in the sample buffer containing 0.0625 M Tris-Cl, pH 6.8, 2% SDS, and 10% glycerol) were separated on 12.5% SDS-PAGE and transblotted onto ECL Hybond nitrocellulose (32) in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3.

RESULTS

Purification of HA-binding Protein and Its Peptide Sequence—34-kDa HA-binding protein was purified from rat kidney tissues (21). Antibodies raised against this purified protein in the rabbit were affinity-purified and used for screening the human skin fibroblast λ gt_{11} cDNA expression library. The peptides generated by partial protease digestion of purified protein were separated by reverse phase high pressure liquid chromatography and subjected to amino acid microsequencing. An amino acid sequence of 14 peptides including the N terminus of the purified protein was determined (Fig. 1).

Cloning and cDNA Sequence of the HA-binding Protein—Using anti-HA-binding protein antibodies as probe, a clone with a cDNA insert (~0.9 kb) was obtained by screening a human skin fibroblast λ gt_{11} expression library. This 0.9-kb cDNA insert was PCR-amplified, and then a 0.4-kb PCR product was sequenced. As shown in Fig. 2*A*, the deduced amino acid sequence matches with the amino acid sequence of peptides obtained from partially digested purified HA-binding protein. Interestingly, a PIR data base sequence search using the FASTP program revealed that the amino acid and cDNA sequence of HA-binding protein match perfectly with the sequence of P-32 reported earlier by Krainer *et al.* (24), followed by Honore *et al.* (35). This protein is co-purified with human HeLa cell pre-mRNA splicing factor SF2 (24, 25), but the functional role of P-32 is not clear. The sequence homology encour-



B

Peptide	Amino acid Sequence
D2	L H T D G
D3	D E I K
D4	D C H Y P E
D5	D S E W R
D6	D T N Y T L N T
D7	D R G V D N T
D8	D E I K E E K X I Q K H K
D9	D L K S F V K S Q
D10	D T N Y T L N T D S L
D12	D W A L Y X K
D13	D G E E E P S Q G Q K A E E
D14	D I F S I K E V S F Q T
D15	D K A F V E F L T
D17	D E L V E L S T A L E H Q E Y I X F

FIG. 1. **Peptide sequence of rat kidney 34-kDa HA-binding protein.** *A*, High pressure liquid chromatogram of HA- binding protein derived peptides. Peptides D_2–D_{17} of HA-binding protein are shown. The CH_3CN elution gradient in 0.1% trifluoroacetic acid is shown by the *straight line*. *B*, amino acid sequence of HA- binding protein peptides separated in *panel A*. Standard amino acid code is used. *X* represents an undetermined amino acid. Peptide D2 constitutes the N terminus of the HA-binding protein.

aged us to sequence the rest of the 0.9-kb cDNA of HA-binding protein with the help of two specific primers made from P-32 cDNA sequence (24). Our 852-nucleotide long partial cDNA sequence of fibroblast HA-binding protein is shown in Fig. 2*A*. This includes a 591-nucleotide coding region of open reading frame and 3′-untranslated region that is 239-nucleotides long (excluding the poly(A) tail) containing potential polyadenylation signals (AACAAA, CATAAA) with some homology to the consensus polyadenylation signals but not an exact match. Moreover, it is shown that deduced amino acid sequence is identical to the amino acid sequence derived from peptides of protease-digested purified HA-binding protein, thus confirming the authenticity of the clone.

Homology of HA-binding Protein Sequence with P-32—The partial cDNA sequence of HA-binding protein is completely identical to the P-32 sequence published by Krainer *et al.* (24) and Honore *et al.* (35). The 3′-untranslated region (239 nucleotides excluding the poly(A) signal) is in agreement with the reported sequence of Honore *et al.* (35), but in Krainer's P-32 it further extends up to 392 nucleotides. Krainer's group viewed P-32 as a full mature protein of 209 amino acids with a CTG (Leu) initiation codon. But Honore *et al.* (35) observed that P-32 is produced as a proprotein of 282 amino acids with a conventional ATG start codon. Honore *et al.* further demonstrated that the 209-amino acid protein demonstrated by Krainer's group is the mature functional protein that is produced by post-translational processing of 282-amino acid-long proprotein. Recently, Luo *et al.* (36) have isolated a murine protein YL2 with 92% sequence homology with the mature P-32, which modulates the function of human HIV type 1 Rev. Our observations that the composite open reading frame of HA-binding protein obtained from N terminus data and cDNA sequence represents the mature protein of 209 amino acids is in agreement with the observations of all the above groups (Fig. 2, *A* and *B*).

Homology of P-32 or HA-binding Protein with CD44—The amino acid sequence of P-32 or HA-binding protein was aligned with other HA-binding protein sequences. At the N-terminal region of mature protein (amino acids 74–161), 14 amino acids out of 88 possible matches were found conserved without any break with the transmembrane and cytoplasmic domain of CD44, the well known HA receptor (Fig. 3).

P-32 Is Antigenically Similar to HA-binding Protein—In order to demonstrate the functional identity of P-32 protein, P-32 cDNA was overexpressed in *Escherichia coli*, and recombinant protein in the cell extract was detected by the antibodies raised against HA-binding protein. As shown in Fig. 4*B*, a single band of 34 kDa was immunodetected in induced cell extracts (*lane 4*), confirming that P-32 protein and the HA-binding protein are homologous proteins.

Purification of P-32 Recombinant Protein by Single Step HA-Sepharose Affinity Chromatography—The recombinant P-32 was purified (Fig. 5*A*) using the single step HA-Sepharose affinity column used for purification of HA-binding protein. The mobility of the recombinant P-32 protein is the same as that of the purified HA-binding protein on SDS-PAGE (Fig. 4*A*), but on SDS-PAGE the movement of recombinant P-32, which should actually show a molecular mass of around 24 kDa, is anomalous as already observed by Krainer *et al.* (24). The isoelectric point (pI) of recombinant P-32 determined by two-dimensional gel electrophoresis (Fig. 5*B*) is in conformity with the theoretical pI of 4.04.

Immunodetection of HA-binding Protein with Anti-P-32 Antibodies—To study the antigenic nature of P-32, the polyclonal antibodies were raised against the purified recombinant P-32. As shown in Fig. 6*A*, both the purified P-32 and the HA-binding protein can be detected by anti-P-32 antibodies. For further confirmation, the P-32 antibodies used in this blot were removed, the blot was reprobed with anti-HA-binding protein antibodies, and the same band could be revisualized (Fig. 6*B*).

Specific Affinity of P-32 toward HA—In order to determine if P-32 protein binds specifically to HA, varying concentrations of purified recombinant P-32 were dot-blotted on Hybond nitrocellulose filters and probed with biotinylated HA. As shown in Fig. 7*A* (*blot D*), the binding of P-32 protein to biotinylated HA increases with the concentration of P-32. The binding of P-32 to biotinylated HA is blocked with excess (20-fold) unlabeled HA (*blot C*) showing the specific affinity of P-32 protein to HA. Moreover, the specific binding of HA to P-32 cannot be competed with a 20-fold excess of RNA (*blot B*) and DNA (*blot A*), ruling out the possibility that P-32 is an RNA or DNA binding protein. The specific binding of HA takes place with P-32 protein in both reducing (with β-mercaptoethanol, *slot 1*) and nonreducing conditions (without β-mercaptoethanol, *slot 2*) in biotinylated HA transblot experiment (Fig. 7*B*).

DISCUSSION

We have described here the cloning of the partial cDNA clone for 34-kDa HA-binding protein and confirmed this protein as P-32, a protein that co-purified with pre-mRNA splicing factor SF2 from human HeLa cells (24, 25). The conclusion is based on the following facts: first, both proteins, namely P-32 and HA-binding protein, are completely identical at cDNA levels; secondly, the predicted amino acid sequence from the cDNA sequence of P-32 protein (24) is again almost identical with

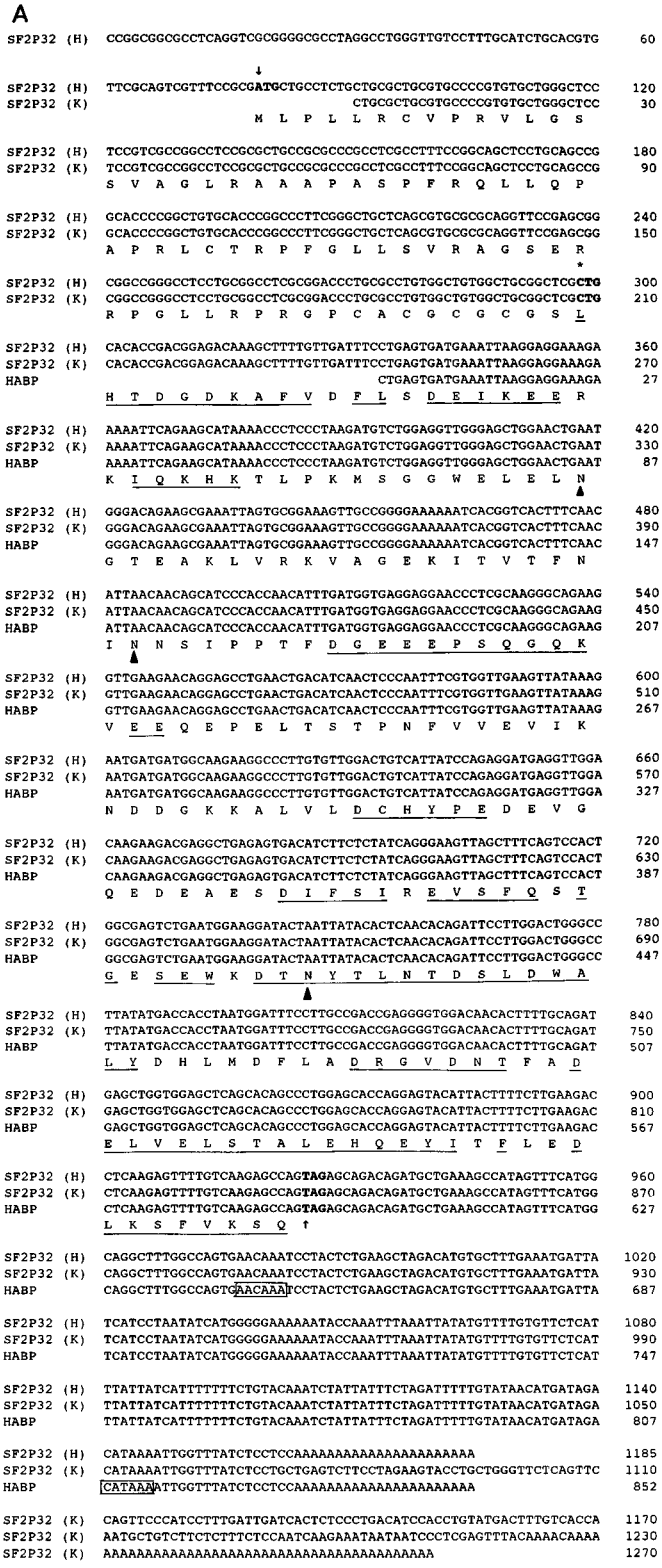


FIG. 2. *A*, triple alignment of cDNA and predicted amino acid sequences of SF2P-32 as published by Honore *et al.* (35) and Krainer *et al.* (24) and HA-binding protein in this study. The one-letter amino acid notation is used. The *inverted* and *upright arrows mark* the initiation and termination codon, respectively. Potential polyadenylation signals in HA-binding protein cDNA sequence are *boxed*. The amino acid sequences derived from purified HA-binding protein by peptide sequencing are *underlined*. *Closed triangles* indicate potential *N*-glycosylation sites. The *asterisk* marks the initiation of mature protein. SF2P32 (*H*) and SF2P32 (*K*) denote the sequence of P-32 published by Honore *et al.* (35) and Krainer *et al.* (24), respectively. *B*, schematic diagram of P-32 sequence published by Honore *et al.* (35) (*a*) and Krainer *et al.* (24) (*b*) and of HA-binding protein in this study (*c*). The *boxed region* in *part a* represents a proprotein that is post-translationally processed by removal of 73 amino acids at the N terminus (*dotted box*) to produce a mature protein of 209 amino acids (*shaded box*). The N terminus of mature HA-binding protein (*black region*) in *part c* as determined by peptide sequencing of purified HA-binding protein is missing in the partial cDNA sequence. *Diagram* is not drawn to scale.

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HABP   74 L H T D G D K A F V D F L S D E I K E E R K I Q K   98
CD44  273 L A S L L A L A L I L A V C I A V N S R R R C G Q   297

HABP   99 H K T L P K M S G G W E L E L N G T E A K L V R K  123
CD44  298 K K L V I N S G N G A V E D R K P S L N G E A S    322

HABP  124 V A G E K I T V T E N I N N S I P P T F D G E E E  148
CD44  323 K S Q E M V H L V N K E S S E T P D Q F M T A D E  347

HABP  149 P S Q G Q K V E E Q E P E                        161
CD44  348 T R N L Q N V D M K I G V                        360
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FIG. 3. **Alignment of amino acid sequence of SF2P-32 or HA-binding protein with that of CD44.** The N-terminal region (amino acids 74–161) of mature P-32 or HA-binding protein matched with the transmembrane and cytosolic domain of CD44 (amino acids 273–360) without any break. *Box* denotes conserved amino acid.

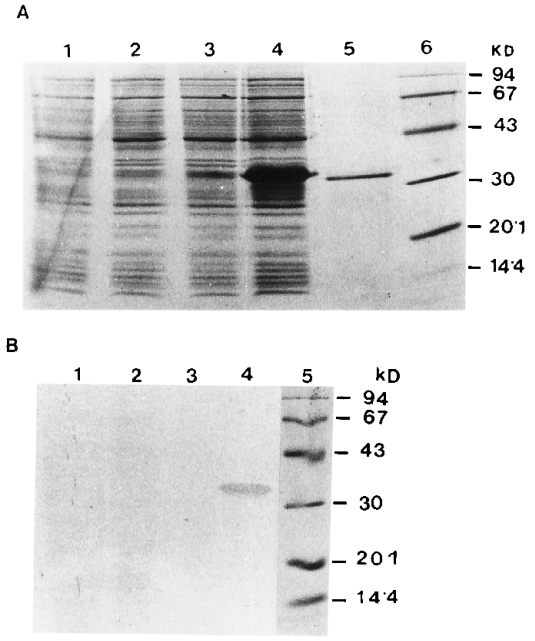

FIG. 4. **Electrophoretic mobility and immunodetection of recombinant P-32 shown by 12.5% SDS-PAGE (*A*) of bacterial (BL21(3E)) lysate containing uninduced pET3c plasmid (*lane 1*, 10 μg), IPTG-induced pET3c plasmid (*lane 2*, 10 μg), uninduced expression plasmid with P-32 cDNA insert (*lane 3*, 10 μg), IPTG-induced expression plasmid with P-32 cDNA insert (*lane 4*, 18 μg), or tissue-derived HA-binding protein (*lane 5*, 4 μg).** Molecular mass markers are included at the *right*. *B*, Western blot analysis of a similar gel, electroblotted on nitrocellulose and probed with rabbit anti-HA-binding protein antibodies. *Lanes 1–4* contain equal amounts of the same proteins as in *panel A*. *Lane 5* indicates molecular mass markers.

partial peptide sequences of purified HA-binding protein; and finally, the identity of P-32 protein as HA-binding protein is confirmed by the presence of known HA-binding motif, purification of recombinant P-32 protein using HA-Sepharose affinity chromatography, the identical electrophoretic mobility, immunocross-reactivity of P-32 protein with anti HA-binding protein antibodies or vice-versa, and the specific affinity of P-32 toward HA.

The functional characteristics of P-32 are not yet known. Krainer *et al.* (24) reported the sequence of P-32 cDNA, which does not contain any conventional ATG (Met) start codon but initiates with a CTG (Leu) codon. The matching of predicted polypeptide sequence with the N terminus of HeLa cell purified P-32 and the absence of upstream ATG codon or consensus 3′ splice sites perhaps had led them to conclude that the mature N terminus does not arise by proteolytic cleavage of a precursor. Later, Honore *et al.* (35) cloned and expressed the cDNA encoding P-32, which extends beyond the 5′ end of cDNA previously reported by Krainer *et al.*, showing that ATG is the start codon at nucleotides 79–81, which is 12 nucleotides upstream of the 5′ end of the sequence published by Krainer *et al.* However, the N terminus sequence of P-32 protein synthesized by cells infected with the Vaccinia virus construct of full-length cDNA including the conventional ATG start codon gave the N terminus amino acid sequence of P-32 as reported by Krainer *et al.*, clearly defining the synthesis of P-32 as a proprotein of 282 amino acids, which is post-translationally processed by removal of the initial 73 amino acids to a mature protein of 209 amino acids. From our study, it is clear that the composite amino acid sequence of HA-binding protein containing 209 residues is completely identical to that of mature P-32 protein reported by both groups (24, 35).

Celis *et al.* (37, 38) reported that the synthesis of P-32 protein is approximately 2-fold up-regulated in SV40-transformed human keratinocytes and MRC-5 fibroblasts, as compared with their normal counterparts, implying some transformation-dependent role of this protein. Our previous report has also shown a higher expression of HA-binding protein in AK-5, a histiocytic tumor cell line (22). Recently, P-32 has been found to have 92% identity with a murine protein YL2 that modulates the effects of human immunodeficiency virus type 1 Rev (36).

The open reading frame of the mature HA-binding protein predicted the protein as a highly acidic one with 28 glutamic acid (13.3%) and 20 aspartic acid (9.5%) residues having a molecular mass of 24 kDa. However, both the recombinant P-32 and HA-binding protein migrate with the same electrophoretic mobility of 34 kDa (Fig. 4*A*). This ambiguity may be explained due to its highly acidic nature, as an overestimation of molecular masses of highly acidic/charged proteins in SDS-PAGE is known (39, 40). The predicted pI from the amino acid sequence is 4.04 (24), which has been also verified experimentally (Fig. 5*B*).

The mature HA-binding protein showed several interesting features. The deduced amino acid sequence of the protein demonstrated the presence of a motif, minimally required for binding to HA, referred to as BX_7B, where B is either R or K and X_7 is a stretch of seven basic residues in between (10). This protein also has one such site ^{119}KLVRKVAGEK^{128}, which contains one extra glutamic acid residue. Peptide mimicry experiments (10) suggest that internal residues can vary by one amino acid without having a detrimental effect on the ability of the protein to bind HA. The presence of glutamic acid is also reported in the HA-binding motif of TSG-6, a known HA-binding protein (41).

Experimentally, the specific affinity of recombinant P-32 to HA was shown by its binding to biotinylated HA in a concentration dependent manner which can be competed only with excess unlabeled HA but not by RNA or DNA, ruling out its nonspecific anionic interactions. It may be mentioned here that though P-32 is co-purified with pre-mRNA splicing factor, Krainer *et al.* have already demonstrated that P-32 does not bind to RNA (24). Furthermore, general RNA-binding motifs RNP1 and RNP2 are also absent in P-32 although present in the associated protein SF2. The observation that P-32 binds to HA in both reducing and nonreducing conditions suggests the lack of conserved cysteine residues in this protein in contrast to CD44 and cartilage link protein (42–44). Rather P-32 behaves like RHAMM, which also lacks conserved cysteine residues and binds HA equally well under reducing conditions (45).

Search analysis further reveals the presence of a potential tyrosine sulfation site (^{185}DCHY*PEDEV^{193}) and three *N*-glycosylation sites (^{109}WELELN*GTEA^{118}, ^{131}VTFNIN*NSIPPTFD^{144}, and ^{218}EWKDTN*YTLNT^{228}) in the predicted amino acid sequence. Incorporation of radiolabeled sulfate in CD44, a well known HA receptor, is already reported (46, 47). Moreover, this protein has a proline-directed ^{160}PELTSTP^{166} sequence,

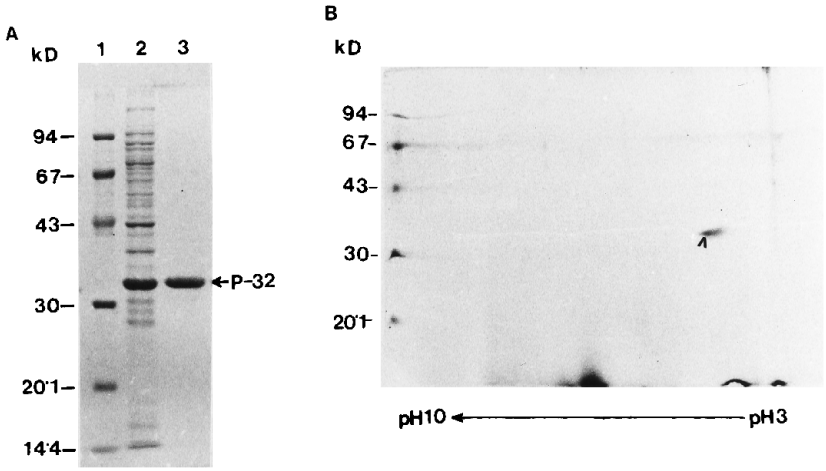

FIG. 5. **Purification profile and two-dimensional gel electrophoresis analysis of recombinant P-32.** *A*, SDS-PAGE of IPTG-induced bacterial extract containing recombinant P-32 protein (*lane 2*, 10 μg) and HA-Sepharose affinity-purified recombinant P-32 (*lane 3*, 5 μg). Low molecular mass markers were loaded in *lane 1*. *B*, two-dimensional gel electrophoresis analysis of purified recombinant P-32. HA-Sepharose column eluate (3 μg of protein) was subjected to isoelectric focusing over a pH range of 3.0–10.0. The focused proteins were separated by 10% SDS-PAGE and stained with Coomassie Blue. An *arrowhead* denotes the P-32 recombinant protein band. Size markers are included on the *left*.

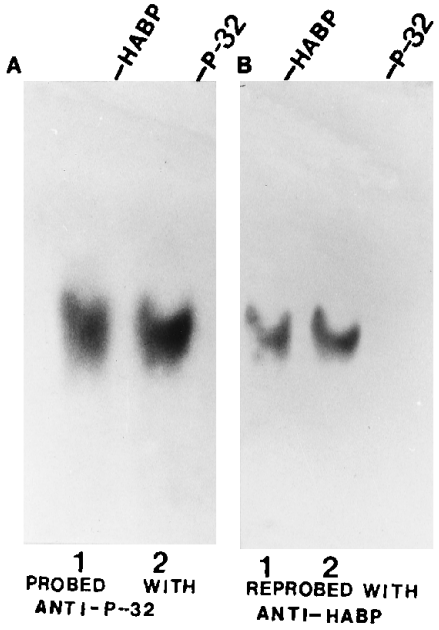

FIG. 6. **Immunocross-reactivity between P-32 and HA-binding protein.** *A*, purified HA-binding protein (*lane 1*, 5 μg) and control purified recombinant P-32 protein (*lane 2*, 5 μg) were transblotted on nitrocellulose and first detected with the anti-P-32 antibodies. *B*, after stripping off the anti-P-32 antibodies, the same blot containing HA-binding protein (*lane 1*) as control was reprobed with anti-HA-binding protein antibodies following the protocol described in the ECL kit.

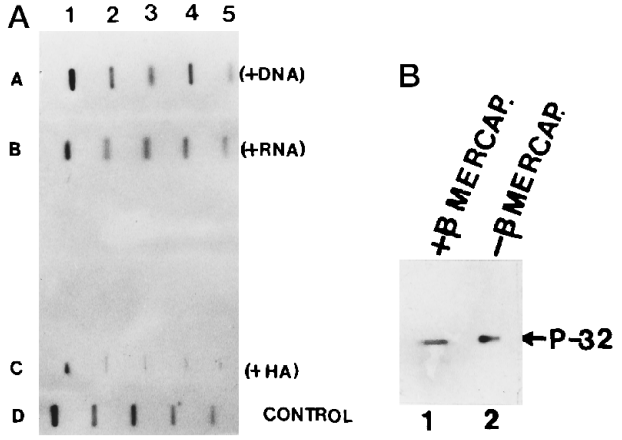

FIG. 7. *A*, specific affinity of recombinant P-32 toward HA. Purified HA-binding protein as control (*slot 1*, 10 μg) and recombinant P-32 (*slot 2*, 8 μg; *slot 3*, 6 μg; *slot 4*, 4 μg; *slot 5*, 2 μg) dot-blotted on nitrocellulose was stained with biotinylated HA (1:4000 dilution) that had been previously blocked with milk protein for 2 h (*blot D*). The other three replicate blots containing the same amount of the proteins are stained in parallel with the same dilution of biotinylated HA in presence of 20-fold excess of unlabeled HA (*blot C*), liver total RNA (*blot B*), and calf thymus DNA (*blot A*). The amount of biotinylated HA bound to the proteins has been detected using avidin peroxidase (1:1500 dilution) and the ECL detection system. Only unlabeled HA blocked binding of HA-binding protein and P-32 to biotinylated HA. Succinic dehydrogenase has also been used as negative control in the blot, which did not stain with biotinylated HA (data not shown). *B*, biotinylated HA transblot assay: Recombinant purified P-32 (5 μg in each *lane*) was subjected to SDS-PAGE under reducing (+β-mercaptoethanol, *lane 1*) and nonreducing conditions (−β-mercaptoethanol, *lane 2*), electroblotted on nitrocellulose, and probed with biotinylated HA as described under "Experimental Procedures."

which may act as the substrate phosphorylation site of protein kinases like extracellular signal-regulated kinase (ERK) (48) and cdc2 family (49). It is already shown that the Ser/Thr-Pro motif is sufficient for phosphorylation by ERK, and the presence of an N-terminal proline residue at least 1 amino acid distant from the phosphorylation site in the motif increases the efficiency of substrate recognition (50).

Sequence data further identifies the mature protein as a multisite-phosphorylated protein, as it has one protein kinase C phosphorylation site (^{202}DIFS*IREVS^{210}) and five casein kinase II phosphorylation sites (^{74}LHT*DGDKAFVD^{84}, ^{200}ESDIFS*IREV^{209}, ^{208}EVSFQS*TGESEWKD^{221}, ^{246}RGVDNT*FADELVEL^{259}, and ^{256}LVELST*ALEHQEYI^{269}). Several protein substrates of casein kinase II have already been identified as substrates of cdc2 kinase (51), supporting our search data.

The presence of five casein kinase II phosphorylation sites in the predicted sequence of HA-binding protein or P-32 protein may further explain the association of P-32 to splicing factor SF2 in HeLa cells. Casein kinase II is ubiquitously present in cytosol and the nucleus of eukaryotic cells (52–54) and can also behave as RNA-binding protein kinase (55). The C group hnRNP protein, implicated in splicing is known to get phosphorylated *in vivo* by a casein kinase II activity (56, 57). Besides C group hnRNP, other pre-mRNA binding proteins, mainly UA2F, the M_r 52,000 protein of trimeric $U_4/U_6/U_5$, and SF2, are

phosphoproteins (58). Mayrand *et al.* (58) proposed that a cascade of critical phosphorylation and dephosphorylation directs their sequential binding and release for participation in the precatalytic state of splicing reaction. In the context of complex association of SF2 and P-32 or HA-binding protein and the presence of multiple casein kinase II phosphorylation sites in P-32, we assume that phosphorylation in this protein may be regulating the splicing ability of SF2. Krainer *et al.* (24) did not rule out the possibility of a role of P-32 in splicing and rather have shown the *in vitro* interaction between P-32 and splicing factor SF2.[2] This observation has recently been supported by the Peterlin group (36) confirming the interaction of YL2 or P-32 with the basic domain of HIV type I Rev, which is important not only for its binding to Rev response element but also for its effect on RNA splicing *in vitro*. In light of the new identity of P-32 as an HA-binding protein, further investigations can be made to explain its association with splicing factor SF2 as well as its role in modulation of the function of human immunodeficiency virus type 1 Rev.

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