Expression Cloning and Molecular Characterization of HAS Protein, a Eukaryotic Hylauronan Synthase*

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We developed a mammalian transient expression system to isolate cDNA clones that determine hyaluronan expression. HAS−, a mouse mammary carcinoma mutant cell line, which is defective in hyaluronan synthase activity, was first established and used as a recipient for the expression cloning. One cloned cDNA that overcame the deficiency was isolated. The cDNA termed HAS contains an open reading frame of 1749 base pairs encoding a new protein of 583 amino acids. Homology analysis of the amino acid sequence suggests that HAS protein is related to streptococcal hyaluronan synthase and also to Xenopus laevis DG42 protein that was found to be homologous to bacterial hyaluronan synthase. Expression of HAS cDNA in HAS− cells complemented not only their mutant phenotypes such as deficient hyaluronan-matrix deposition but also hyaluronan synthase activity itself. Therefore, HAS cDNA is responsible for the activity of the hyaluronan synthase, a key enzyme of hyaluronan synthesis in eukaryotic cells.

Hyaluronan, a high molecular weight linear glycosaminoglycan, which is composed of β1,4-linked repeating disaccharides of glucuronic acid β1,3-linked to N-acetylgalosamine, is a characteristic component of the extracellular matrix during early stages of morphogenesis, and its synthesis is spatially and temporally regulated (1). The association of hyaluronan with the cell surface can influence the cellular behaviors especially in regard to modulation of cell migration, adhesion, wound healing, and tumor invasion (2–5). We are interested in the molecules involved in the association and have found that the heavy chain of the interglobular trypsin inhibitor and PG-M/versican play important roles in the formation of the hyaluronan matrix (6–8). The molecular cloning of genes encoding enzymes that take part in the hyaluronan biosynthesis is one of essential steps to understand the biosynthetic pathway as well as to investigate biological functions of the pericellular association of hyaluronan. Although the hyaluronan biosynthesis in Group A Streptococci has been extensively studied and the structural gene for the bacterial hyaluronan synthase was recently isolated from Streptococcus pyogenes (9), little is known about the mechanism for the biosynthesis of hyaluronan in eukaryotic cells. There are some attempts to purify eukaryotic hyaluronan synthase to a homogeneity. However, those encountered the loss of enzyme activity (10–12). Therefore, we chose the different way by adopting a mammalian transient expression system to isolate the genes responsible for the expression of hyaluronan. We first established several mutants that are defective in hyaluronan biosynthesis. One of the mutant cell line, HAS−, is defective in hyaluronan synthase activity and may therefore be useful to identify a gene encoding eukaryotic hyaluronan synthase. In this report, we describe the isolation of a cDNA encoding a protein that may correspond to hyaluronan synthase in mouse mammary carcinoma cells by a mammalian transient expression cloning.

EXPERIMENTAL PROCEDURES

Materials—UDP-GlcNAc was purchased from Sigma. UDP-[14C]GlcNAc (285.2 mCi/mmol) was purchased from DuPont NEN. Streptomyces hyaluronidase was obtained from Seikagaku Corp., Tokyo, Japan. Sheep fixed erythrocytes were purchased from Inter-Cell Technologies, Inc. Streptomyces hyaluronidase was obtained from Seikagaku Corp., Tokyo, Japan. Superdex HR 10/30 column was purchased from Pharmacia Biotech, Tokyo, Japan.

RESULTS

To obtain G418-resistant and blastici- din S-resistant cell lines, respectively, either pSV2neo vector (Clontech Laboratories Inc.) or pSV2bssr vector (Funakoshi Co., Ltd., Tokyo, Japan) was transfected to each mutant cell line by lipofection as described previously (13). A clone having high capacity of hyaluronan product, FM3A HA1, was established from FM3A P15A by the selection using fixed erythrocyte exclusion assay. To obtain mutant cells with defects in hyaluronan biosynthesis, FM3A HA1 cells were treated with 0.5 μg/ml MNNNG and selected as described above. One of the mutant cell lines, HAS−, is defective in a hyaluronan synthase activity (see “Results and Discussion”). Polysoma large T antigen-expressing cell lines were established by transfecting HAS− mutant cells with pdl3027 plasmid containing polsoma T antigen gene as described previously (14). Polysoma large T antigen-mediated replication of plasmids in these cell lines was assessed by measurement of the methylation status of the plasmid DNA (15). Finally, a clonal cell line named HAS P was chosen for a transient expression cloning.

Complementation Analyses—To obtain G418-resistant and blastici- din S-resistant cell lines, respectively, either pSV2neo vector (Clontech Laboratories Inc.) or pSV2bssr vector (Funakoshi Co., Ltd., Tokyo, Japan) was transfected to each mutant cell line by lipofection as described below. The stable transfecants were selected in the MEMC medium with 500 μg/ml G418 (Life Technologies, Inc.) or 50 μg/ml blastici- dinS hydrochloride (Funakoshi Co., Ltd.). Somatic cell fusion was performed as described previously (16). The fused cells were selected in the MEMC medium with 500 μg/ml G418 and 50 μg/ml blastici- dinS hydrochloride. Complementation was assessed by the production of hyaluronan by the fused cells, which was determined by the immunoenzyme assay described below.

cDNA Library Screening—A CDNA library, pcDNAI-HA1, was constructed from polyA1 RNA isolated from FM3A HA1 cells and mammal- en expression vector pcDNAI (Invitrogen Co.) as described previously (17). The library comprised 1 × 109 independent colonies when transfected to Esherichia coli MC1061/F3. For the first round of transfection, 20 samples of 1.5 × 108 HAS− cells in 60-mm tissue culture
Plasmid DNA molecules were recovered from the sorted cells by the Hirt procedure (18) and transfected to E. coli MC1061/P3. Plasmid DNA was prepared again and used for another round of screening by the same procedure as described above. After four cycles of transfection and screening, 10 pools prepared to contain 10 colonies each were screened by the expression of hyaluronan. Finally, 10 clones from the one positive pool were randomly selected and screened, and one positive clone, HAS, was isolated (sibling selection) (14).

DNA Sequence and Analysis—The insert from the HAS clone was purified and subcloned into pcDNA3 plasmid vector (Invitrogen Co.). The nucleotide sequence of the isolated DNA was determined by repeated sequencing of both strands of alkaline-denatured plasmid DNA using the DGTP and deazaGTP kits with Sequenase version 2.0 (U.S. Biochemical Corp.). The DNA synthesis was primed by T7, SP6, and internal primers situated about 250 base pairs apart. The obtained DNA sequences were compiled and analyzed using GENETYX-MAC computer programs (Software Development Co., Ltd., Tokyo, Japan). The nucleotide and deduced amino acid sequences were compared with other protein sequences in the nucleic acid and protein data bases (EMBL-GDB, release 44 and NBRF-PDB, release 45).

Establishment of Stable Transfectants—pcDNA3-HAS plasmid was prepared as described above. HAS cells were transfected either with pcDNA3-HAS or with pcDNA3 control vector by the lipofection procedure and then selected in the medium with 500 μg/ml G418. Clonal cell lines were obtained by limiting dilution.

Fixed Erythrocyte Exclusion Assay—The fixed erythrocyte exclusion assay followed a protocol described previously (19). Cells were observed under an Olympus IMT-2 inverted phase-contrast microscope.

Cloning of Eukaryotic Hyaluronan Synthase Gene—Crude membrane proteins were prepared and suspended in 0.2 ml of 25 mM Hepes-NaOH, pH 7.1, 5 mM dithiothreitol, 15 mM MgCl₂, 1 mM UDP-GlcNAc, 0.05 mM UDP-GlcUA, 2.5 μCi of UDP-[14C]GlcUA as described previously (11). However, the substrate concentrations in the reaction mixture were modified to give the maximal activity from the original ones. Protein content was determined using the Bio-Rad protein assay kit. Hyaluronan synthase activity was assayed by the procedure modified from that of Tengblad (20). We used b-HABR and alkaline phosphatase-conjugated streptavidin as primary and secondary probes, respectively. The enzyme activity was measured using p-nitrophenyl phosphate as a sensitive substrate.

Hyaluronan Synthase Assay—Cultures were washed with cold Cosmedium 001 and resuspended in the culture medium containing fluorescein avidin DCS (Vector Laboratories, Inc.). After incubation for 1 h on ice, the cells were washed and resuspended in cold phosphate-buffered saline, pH 7.4, containing 5% fetal calf serum. Positively stained cells were sorted by EPICS Elite Flow Cytometer (Coulter Electronics, Inc.).

### Table I

| Cell line | HA synthesisa | pmol/h/mg protein |
|-----------|---------------|-------------------|
| FM3A HA1  | 140 ± 8       | 84.6 ± 3.2        |
| HAS       | 5 ± 2         | 0.1 ± 0.0         |
| Fused cells | 73 ± 3   | NDb                |

a The amount of HA in the culture medium was normalized per 10⁴ cells.

b Specific activity, picomoles of glucuronic acid/h/mg of protein. The values given are means ± S.D. for three experiments.

c The nucleotide and deduced amino acid sequences were compared with other protein sequences in the nucleic acid and protein data bases (EMBL-GDB, release 44 and NBRF-PDB, release 45).

d Not determined.
and was termed HAS class C almost lacked hyaluronan synthase activity (Table I). By contrast, the one clone representing the protein, respectively). Mutant cell lines deficient in hyaluronan biosynthesis were isolated from FM3A HA1 cells mutagenized with MNNG. All the mutants showed a considerably reduced level of hyaluronan production (less than 6 ng of HA/10^4 cells) compared with that of the wild-type FM3A HA1 cell line (see Table I). The genetic backgrounds of these clones were analyzed by somatic hybridization (9, 10). The analysis suggested the presence of the hydrophilic region between the NH2-terminal hydrophobic stretches and the COOH-terminal hydrophobic stretches (Fig. 2). In comparison with the hydrophobicity profile of a bacterial hyaluronan synthase, HasA protein (24), highly similar molecular arrangements were observed. Mian (10) described previously that a 66-kDa protein may be a constituent of the membrane-bound hyaluronan synthase complex partially purified from the detergent-solubilized plasma membrane of cultured human skin fibroblasts. Considering these, the structural characteristics of HAS protein suggest that the protein may be either the hyaluronan synthase itself or an essential component of the synthase complex.

RESULTS AND DISCUSSION

Mutant cell lines deficient in hyaluronan biosynthesis were isolated from FM3A HA1 cells mutagenized with MNNG. All the mutants showed a considerably reduced level of hyaluronan production (less than 6 ng of HA/10^6 cells) compared with that of the wild-type FM3A HA1 cell line (see Table I). The genetic backgrounds of these clones were analyzed by somatic cell fusion and resultant complementation in hyaluronan biosynthesis. The clones were found to be grouped into three classes (A, B, and C), and any combination of the clones between the different classes complemented the hyaluronan production (Table I), which suggested that at least three genes may contribute to hyaluronan biosynthesis and that the hyaluronan synthesis would be restored if the normal gene is introduced into the mutant cells. The typical clones representing class A and B maintained significant levels of the hyaluronan synthase activity (45.2 ± 1.5 and 35.9 ± 3.2 pmol/h/mg of protein, respectively). By contrast, the one clone representing class C almost lacked hyaluronan synthase activity (Table I) and was termed HAS− for subsequent study.

To clone a cDNA encoding a protein that participates in hyaluronan synthase, a transient expression cloning using the GENETYX-MAC program. The amino acids displayed correspond to residues 242–391 for HAS, 240–389 for DG42, and 134–372 for HasA proteins. Regions of approximately 150 amino acids were aligned using the GENETYX-MAC program. The amino acids displayed correspond to residues 242–391 for HAS, 240–389 for DG42, and 134–372 for HasA proteins.

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A computer search for proteins having identity to HAS protein was performed using the NBRF-PDB (release 45) database. None of sequences identical to HAS protein was found in the protein database. However, interestingly, a 557-amino-acid overlap with 57.6% identity to HAS protein was observed with DG42 protein, the predicted product of an mRNA species that is rapidly accumulated and degraded during Xenopus laevis embryonic development (25). DG42 protein had also been reported to be a protein homologous to HasA protein (9). The
search also showed significant homology between HAS protein and HasA protein (290-amino-acid overlap with 33.1% identity). DeAngelis et al. (26) recently reported the presence of the conserved region of HasA protein among the various Group A Streptococci which is likely to be involved in the structure and/or function of the hyaluronan synthase. This region is also very similar in GlcNAc polymer synthases such as yeast chitin synthases and Rhizobium NodC (26). Thus, we compared those regions among HAS, DG42, and HasA proteins using the GENETYX-MAC program (Fig. 3). The multiple sequence alignments of the regions showed that there is 76.7% identity between HAS and DG42, and 40.7% identity between HAS and HasA, respectively. The sequence conservation strongly supports that HAS gene product is greatly related to eukaryotic hyaluronan synthase. Although DG42 has recently been reported to synthesize oligosaccharide of GlcNAc but not hyaluronan synthase, membrane fractions of those stable transfectants were assayed for the hyaluronan synthase activity that bearsthe GlcUA transferase activity. The neo-synthesis of hyaluronan by HAS cDNA was confirmed using stable transfectants expressing the HAS gene.

A transient expression of HAS cDNA in HAS- P cells complemented the deficient matrix deposition of hyaluronan (Fig. 4). Pretreatment of the cells with Streptomyces hyaluronidase completely abolished the formations of hyaluronan matrix surrounding the transfectants (Fig. 4D). After hyaluronidase digestion, the areas of the hyaluronan matrix were identical to those observed in control (transfectants with pcDNA1) (Fig. 4C). The neo-synthesis of hyaluronan by HAS cDNA was confirmed using stable transfectants expressing the HAS gene. The cell line established from HAS- cells transfected with pcDNA3-HAS synthesized and secreted hyaluronan at significantly higher levels in the culture medium (Table I). The control transfectants with pcDNA3 vector produced low levels of hyaluronan. These results again support that HAS protein takes part in the essential step of hyaluronan biosynthesis. To obtain evidence for the possibility that HAS cDNA encodes hyaluronan synthase, membrane fractions of those stable transfectants were assayed for the hyaluronan synthase activity as described under "Experimental Procedures." The significant activity was detected in the fractions from the transfectants with HAS cDNA (Table I).

Over all, our data demonstrate that the HAS gene product is responsible for the activity of the hyaluronan synthase and may correspond to synthase itself. Future studies on the activity of recombinant HAS protein will give the final conclusion.

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