Functional Cloning of the cDNA for a Human Hyaluronan Synthase*

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Hyaluronan (hyaluronic acid) is a high molecular mass polysaccharide that has ubiquitous distribution in the extracellular matrix, with highest concentrations in soft connective tissue. It is a linear polysaccharide consisting of alternating glucuronic acid and N-acetylglucosamine residues linked by β-1→3 and β-1→4 glycosidic bonds (1). Hyaluronan has several physiochemical and biological functions such as space filling, lubrication, and providing a hydrated matrix through which cells can migrate (2, 3). Interaction of hyaluronan with the cell surface receptor CD44 has been shown to contribute to organ-specific leukocyte homing and migration (4–8). The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U59269.

EXPERIMENTAL PROCEDURES

Plasmids, Monoclonal Antibodies, and Cell Lines—The following plasmids were used as controls in expression cloning and for functional adhesion assays: pSV-SPORT-1 (Life Technologies, Inc.) or pCDNA3 (Invitrogen, San Diego, CA.) controls and murine MadCAM-1 in pCDM9 (pCDMAD-7 (26)), monoclonal antibodies (mAbs) used were anti-murine CD-44 TJB1.7 (2), anti-murine MAdCAM-1 MECA-367 (27), anti-human VCAM-1 2G7 (28), anti-murine bFib 504 (29), and anti-murine aPS/2 (30). Cell lines used for expression cloning and functional adhesion assays were CHO/P and the murine T cell lymphoma TK1 (31, 32).

cDNA Synthesis and Library Construction—mRNA was isolated from the human mesenteric lymph nodes using standard procedures previously described (26). cDNA libraries were constructed as described (33) with the exception that bacterial pools at a starting density of 5,000 clones/plate were used for the initial screen.

Expression Cloning—CHO/P cells were seeded into 24-well plates approximately 24 h prior to transfection at a density of 40,000 cells/well. Transient transfections and expression cloning were performed as recently described (33).

Functional Adhesion Assays—Assays with purified clones were similar to those performed in expression cloning with the following exception: as several wells were to be transfected for antibody inhibition studies, a master liposome mixture with multiples of the wells to be

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Hyaluronan is a constituent of the extracellular matrix of connective tissue and is actively synthesized during wound healing and tissue repair to provide a framework for ingrowth of blood vessels and fibroblasts. Changes in the serum concentration of hyaluronan are associated with inflammatory and degenerative arthropathies such as rheumatoid arthritis. In addition, hyaluronan has been implicated as an important substrate for migration of adhesion of leukocytes during inflammation. A human hyaluronan synthase (HuHAS1) cDNA was isolated by a functional expression cloning approach. Transfection of CHO cells conferred hyaluronidase-sensitive adhesiveness of a mucosal T cell line via the lymphocyte hyaluronan receptor, CD44, as well as increased hyaluronan levels in the cultures of transfected cells. The HuHAS1 amino acid sequence shows considerable homology to the hasA gene product of Streptococcus pyogenes, a glycosaminoglycan synthetase from Xenopus laevis (DG42), and is the human homolog of a recently described murine hyaluronan synthase.

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*The abbreviations used are: RHAMM, receptor for hyaluronan-mediated motilility; mAb, monoclonal antibody; PBS, phosphate-buffered saline; HBSS, Hank's balanced salt solution; UDP-[3H]GlcNAc, UDP-[14C]GlcUA, UDP-N-acetylglucosamine; UDP-[14C]GlcUA, UDP-glucuronic acid.

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transfected was first made for each plasmid. For mAb inhibition studies, mAbs were incubated with cells at 10 μg/ml at 4 °C for 15 min prior to the start of the assay.

For adhesion assays with hyaluronan, human umbilical cord hyaluronan (Calbiotech) was diluted to 5 mg/ml in PBS. Streptomyces hyaluronidase (Calbiochem) was diluted to 20 turbidity reducing units/ml in HBSS. TK1 cells were suspension maintained in HBSS containing 2 mM CaCl2 and 2 mM MgCl2, 3% fetal calf serum, and 20 mM HEPES at 106 cells/ml. Wells of 24-well plates were coated with 200 μl of hyaluronan and stored at 4 °C overnight. Wells were rinsed with 0.5 ml of PBS three times and treated with 0.25 ml of Streptomyces hyaluronidase at final concentrations of 0, 5, 10, and 20 turbidity reducing units/ml for 1 h at 37 °C. Wells were rinsed three times with 0.5 ml of PBS, blocked with 0.5 ml of fetal calf serum for 1 h on ice, and then rinsed three times with 0.5 ml of PBS. TK1 cells (0.5 ml) were added to each well, and plates were incubated with shaking at 4 °C for 20 min.

For assessment of hyaluronate-mediated binding to CHO/P cells, the transfectants were rinsed with 0.5 ml of PBS three times. Individual wells were treated with 250 μl of Streptomyces hyaluronidase at 0, 5, 10, and 20 turbidity reducing units/ml (final concentrations) for 1 h at 37 °C. Transfectants were rinsed three times with 0.5 ml of PBS. TK1 cells (0.5 ml in the same buffer as described above) were added to each well, and plates were incubated with shaking at 4 °C for 30 min. Wells were rinsed with 0.5 ml of PBS three times and viewed under the light microscope. For each assay, the number of TK1 cells bound per CHO/P cell was averaged for a minimum of four fields (20× objective) with standard deviation. Assays reported were from one of four representative experiments, all with similar results.

Measurement of Hyaluronic Acid Biosynthesis in CHO Cell Transfectants—0.5 × 106 CHO cells seeded in 100-mm plates were transfected with LipofectAMINE™ reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. Transfections utilized 20 μg of HAS cDNA in pDEN3 (Invitrogen) and 180 μl of LipofectAMINE™ reagent. Approximately 72 h after transfection, 440 μg/ml G418 was added in fresh media. After the transfected and control (nontransfected) cells had reached subconfluency, the medium was replaced with fresh complete media containing 5 μCi/ml of 3H-glucosamine hydrochloride (DuPont NEN, specific activity 33.3 Ci/ml, concentration 1 μCi/ml), a precursor of both sulfated glucosaminylglycans and hyaluronan. The amounts of synthesized hyaluronan in transfected and control CHO cells were determined after 48 h of incubation at 37 °C as follows. Media were collected and the cell layers were dissolved in 0.06 N NaOH for 30 min, followed by neutralization with HCl, and combined with the corresponding media. Aliquot from each sample was incubated overnight at 37 °C in the presence or absence of Streptomyces hyaluronidase (10 units/ml). Then the samples were applied on a Sephadex G-50 superfine column (50 × 100 mm) which was equilibrated with 0.05 M sodium acetate, pH 6.0, containing 0.02 M NaCl. The Vc and V0 of the column were determined by chromatography of [3H]hyaluronan of Mw = 970,000 (tritium hyaluronan labeled in acetyl groups, 38 μCi/ml, 14 × 106 dpm/ml which was a generous gift of J. R. E. Fraser, Melbourne, Australia) and [3H]O (Amersham Life Science), respectively. Newly synthesized [3H]hyaluronan was determined as the Streptomyces-sensitive radioactivity eluting in the void volume. Hyaluronan production was also determined with a commercially available kit (HA-test 50, Pharmacia Diagnostica, Uppsala, Sweden).

DNA Sequencing—Plasmids were sequenced on both strands using oligonucleotide primers and the Sequenase77 T-deaza-dGTP DNA sequencing kit with Sequenase version 2.0 T7 DNA polymerase (United States Biochemical) and [35S]dCTP (Amersham Life Science and DuPont NEN) using manufacturer’s instructions.

Northern and Southern Blot Analysis—Northern and Southern blots were human multiple tissue Northern and II (Clontech). Hybridization, radioactive probe preparation, and film exposure was performed as described previously (33) (as was for a commercially prepared Southern blot (Human GENO-BLOT (Clontech)).

RESULTS AND DISCUSSION

An expression cloning system was developed to isolate cDNA clones that encode proteins that confer adhesion of the murine mucosal homing T cell lymphoma TK1 (32). A human mesenteric lymph node expression library was constructed that, upon transfection into CHO/P cells, yielded a cDNA clone, called 30C, that mediated significant TK1 cell rosetting. The adhesion assay was then repeated after preincubation with several mAbs to adhesion receptors known to be expressed on TK1 cells. Binding could be completely inhibited by preincubation of TK1 cells with an antibody to CD44 (Fig. 1A), while other mAbs (anti-β7- and anti-α4-integrin (29, 30)) had no effect. As CD44 is known to be a hyaluronan receptor (5–7), we wanted to know
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Fig. 2. Nucleotide and deduced protein sequence of HuHAS1 cDNA. Cysteine residues are circled, and a conserved motif, B\(\times\)X\(2\)B, believed to be important for binding hyaluronan, is lightly outlined. Consensus phosphorylation sequences for protein kinase C (RHLT, KYT, and RWLS) and cAMP-dependent protein kinases (RWS) are outlined in bold. Also shown with a bold underline at position 2066 is a consensus polyadenylation signal, AATAAA.

if the isolated cDNA encoded a novel CD44 ligand or, alternatively, was involved in de novo synthesis of hyaluronan. Hyaluronidase pretreatment completely abrogated TK1 binding to transfected CHO cells (Fig. 1 A). Furthermore, hyaluronan production, as measured with a commercially available kit, was 3-fold higher in CHO cells transfected with 30C cDNA than control transfected (not shown).

The cDNA encoding clone 30C is 2116 nucleotides in length (Fig. 2) with a short 5′-untranslated region of 39 bp and a longer 3′-untranslated region of 416 bp. From the first ATG we predict an open reading frame of 1734 bp which yields a protein of 578 amino acid residues. GenBank searches of the nucleotide and protein sequences revealed significant homology with the hasA gene of S. pyogenes (21), which was reported to be a hyaluronan synthase, a X. laevis cDNA (DG42) (Fig. 3, A and B) which also appears to be a glycosaminoglycan synthetase (23, 24) and a recently isolated murine hyaluronan synthase cDNA (25, 33). Amino acid sequence identities between the human cDNA and these sequences were 22%, 54%, and 92%, respectively. Significant similarity was also observed with other membrane-associated proteins with N-acetylgalosaminyltransferase activity including NodC from Rhizobium and three chitin synthases from Saccharomyces (22) (not shown). The similarities observed, coupled with the functional analysis data, led us to conclude that clone 30C encodes the human equivalent of hyaluronan synthase. Using nomenclature based on the Strepotoccus gene locus and the murine protein, we now refer to this human hyaluronan synthase as HuHAS1.

The predicted molecular mass of HuHAS1 is 64,793 daltons. Hydrophilicity (Kyte/Doolittle) analysis suggests a membrane protein with several potential membrane spanning regions (Fig. 3, A and B), consistent with labeling studies which suggests that HuHAS1 is associated with the plasma membrane (19, 34–36). When secondary structure is taken into account, there is considerable conservation between HasA, DG42, and HuHAS1, as all of these proteins have very similar hydrophilicity plots (Fig. 3B). A central hydrophilic region of all of these proteins might be predicted to be a large intracellular loop, also consistent with studies indicating that hyaluronan biosynthesis occurs at the inner surface of the plasma membrane (19, 34). When comparing this region to DG42, we find greater similarity to HuHAS1 than the overall protein at 70% (versus 54%) which would imply conservation of a functional domain. Within the amino-terminal portion of this domain lies a motif, designated B\(\times\)X\(2\)B (Fig. 2), where B is a basic amino acid (either Arg or Lys) and X is any nonacidic residue with at least one more basic amino acid residue. This motif has been found in both RHAMM, link protein, and CD44 and has been shown to be required for binding hyaluronan (37). The presence of this putative hyaluronan binding motif in HuHAS1 raises the possibility of a requirement of binding hyaluronan during its synthesis and prior to transport out of the cell.

Northern blots probed with HuHAS1 cDNA reveal a major transcript of 2.4 kb that is most highly expressed in ovary and also expressed at significant levels in spleen, thymus, prostate, testes, and large intestine (Fig. 4 A). Extremely weak expression was observed in small intestine while peripheral blood leukocytes were negative. Moderate expression was also observed in heart (not shown). In some tissues, larger transcripts were observed (Fig. 4A) that might be a related gene (or unprocessed RNA) although a Southern blot probed first with the full-length and then a 3′ region of HuHAS1 cDNA (and washed at several temperatures) shows a single banding pattern suggestive of a single copy gene (Fig. 4B). The expression pattern observed is consistent with high levels of hyaluronan that are observed in lymphoid tissues, preovulatory follicles, and in perivascular connective tissue and vessel walls of both atrium and ventricle (38, 39) and would indicate that synthesis of hyaluronan is at least partially regulated by transcriptional mechanisms. Expression of HuHAS1 RNA was barely detectable in skeletal muscle (not shown), although histochemical analysis has shown ubiquitous distribution of hyaluronan in connective tissue and the septum dividing muscle fibers (38, 39). Interestingly, another murine hyaluronan synthase has also been isolated (50) which is not identical to the recently
published murine HAS1 sequence and shares only 72% identity with our HuHAS1 protein. This suggests the presence of two murine HAS proteins and raises the possibility that there are two human synthases as well. This finding implies that differential transcriptional regulation of multiple HAS genes could be an additional mechanism of controlling rates of hyaluronan synthesis in various tissues, several inflammatory settings, and during development as well.

Induction of synthase activity by growth factors has also been shown to correlate with phosphorylation events (20, 40, 41). Examination of hydrophilic regions of HuHAS1 reveals several conserved motifs which are potential substrates for protein kinase C and cAMP-dependent kinases (Figs. 2 and 3C) (42). It is likely that the regulation of hyaluronan synthesis is mediated by regulation of HuHAS1 gene transcription, in addition to complex regulatory circuits which involve both alterations in phosphorylation of the synthase or proteins associated with HuHAS1.

Previously, a 52-kDa protein was isolated from a mouse/hamster hybridoma (B6 cells) that was initially reported to be a mammalian hyaluronan synthase (35). This protein was incapable of binding UDP-[14C]GlcA and UDP-[3H]GlcNAc unless complexed to a 60-kDa protein, which may be the hyaluronan receptor (RHAMM) recently implicated in fibroblast migration and tumor metastasis (43). This protein cross-reacted with antibodies against a putative synthase from S. equisimilis. The gene encoding this protein was cloned from a streptococcal library and shown to be related to proteins involved in oligopeptide processing and transport and showed no homology to the hasA gene sequence (36, 44). It is likely that the 52-kDa protein isolated from the B6 line is a homolog to the streptococcal transport protein and not the synthase itself. This human cDNA is therefore one of the first examples of a mammalian gene responsible for synthesis of hyaluronan.

Studies in streptococci show that the machinery responsible for synthesis of hyaluronan is encoded in the has operon which consists of three genes, hasA, hasB, and hasC (45–47). We have demonstrated that HuHAS1 is homologous to hasA which encodes hyaluronan synthase, along with two recently cloned cDNAs encoding two different murine synthases (25) and see accompanying report by Spicer et al. (50). The hasB and hasC loci encode UDP-Glc dehydrogenase and UDP-Glc pyrophos-
phylosylase, respectively (45–47). Transfection of the HuHAS cDNA into CHO cells is sufficient to mediate de novo synthesis of hyaluronan, which indicates that all of the other factors necessary for hyaluronan biosynthesis such as those encoded by hasB and hasC are possibly expressed in CHO cells. Interestingly, recent studies have implied that DG42 might actually encode a chitin synthase that, by synthesis of short chitin oligomers might act as a “primer” for biosynthesis of hyaluronan, and raises the possibility that HuHAS might also act as a chitin synthase (48, 49) and suggests that other synthases must be present for hyaluronan synthesis. This paper and other recent reports, however, collectively show that HAS cDNAs can be expressed in a variety of cellular backgrounds including CHO, COS, mouse mammary carcinoma, rabbit kidney, and human osteosarcoma cells, all resulting in production of hyaluronan (23, 25, 50). It seems unlikely that a chitin synthase activity is missing from all of these backgrounds while at the same time they all contain an inactive hyaluronan synthase activity (in other words, only lacking putative chitin synthases), although this possibility needs further evaluation. An alternate possibility, however, is that the recently cloned synthases are capable of biosynthesis of both chitin and hyaluronan. The identification of HuHAS (and the murine HAS genes as well) will therefore assist further characterization of the molecular events resulting in synthesis of hyaluronan and its relationship to cellular migration in wound healing, tumor metastasis, and leukocyte migration.

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