HAS1 was expressed as a FLAG-tagged HAS1 fusion protein in COS-1 cells. This recombinant protein was extracted with CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid) from the membrane fraction and purified by anti-FLAG affinity chromatography and subsequent SDS-polyacrylamide gel electrophoresis. A protein solubilized from the one single band on the gel was able to synthesize hyaluronan when incubated with UDP-GlcNAc and UDP-GlcA as donor substrates without any further additions. The detergent-solubilized and purified HAS1 protein, however, exhibited quite different kinetic properties from the membrane-bound protein. When assayed under the reconstitutive conditions where the reaction mixture was layered onto the buffer containing high concentration of CHAPS, the activity was enhanced and the kinetic properties became similar to those of the membrane-bound protein. In addition, a HAS1 gene product by an in vitro transcription/translation system also showed HAS1 activity under the reconstitutive conditions. To our surprise, when incubated with UDP-GlcNAc alone, the protein was found to synthesize chito-oligosaccharide. Taking advantage of these enzyme reaction properties, active sites on the protein involved in for hyaluronan and chito-oligosaccharide synthesis were characterized. Site-directed mutagenesis induced in the cytoplasmic central loop domain of the protein revealed that several amino acid residues conserved among those domains of various proteins of a HAS family were essential for both hyaluronan and chito-oligosaccharide syntheses but one of them was not for chito-oligosaccharide synthesis. The substitutions that caused partial or severe loss of the activity gave no significant changes of the $K_m$ values of the mutated proteins, suggesting that no conformational or other indirect changes were involved in the effect. Taken together, the results suggest that the HAS1 protein alone is able to synthesize hyaluronan and different amino acid residues on the cytoplasmic central loop domain are involved in transferring GlcNAc and GlcA residues, respectively.

Hyaluronan (HA) is a high molecular weight linear glycosaminoglycan, which is composed of β-1,4-linked repeating disaccharides of glucuronic acid β-1,3-linked N-acetylgalactosamine. HA is not only an important structural element in cartilage, synovial fluid, vitreous humor of eye, and skin of vertebrates but also may play important roles in many biological processes such as tissue organization, tissue morphogenesis, cancer metastasis, wound healing, inflammation, angiogenesis, etc., which are also evident from the recent observations that the association of HA with cell surface receptors such as CD44 and RHAMM (receptor for hyaluronan-mediated motililty) influences cellular proliferation, differentiation, migration, and adhesion (1-6).

In contrast to a massive accumulation of the findings on structural and physiological significance of HA stated above, no enough information has been available on the mechanism of HA synthesis. Recently, three distinct but highly related genes encoding putative mammalian HA synthases, HAS1, HAS2, and HAS3, have been cloned in mouse and human (7-12). They displayed sequence homology to the Streptococcus pyogenes HA synthase, spHasA, to the developmentally regulated Xenopus laevis DG42 protein, and to a variety of β-glucosaminyltransferases such as the Rhizobium NoD proteins, yeast chitin synthases, and a family of putative plant cellulose synthases. Mammalian HAS proteins play crucial role in the HA biosynthetic pathway, since expression of HAS proteins leads to HA synthesis by transfected mammalian cells (7-12). However, it has remained to answer whether mammalian HAS proteins alone have the ability to synthesize hyaluronan from sugar nucleotide precursors UDP-GlcA and UDP-GlcNAc or whether other protein components are essential for the synthesis.

With regard to a bacterial hyaluronan synthase, DeAngelis et al. (13, 14) showed that the immunoaffinity-purified recombinant spHasA protein synthesized a hyaluronan of high molecular mass with exogenously supplied UDP-GlcA and UDP-GlcNAc. They also demonstrated that the DG42 gene product could synthesize hyaluronan when expressed in yeast, which was shown to have no activity to produce hyaluronan or UDP-GlcA. The membrane extracts of the transfected yeast cells synthesized hyaluronan of a high molecular mass in the presence of the exogenously supplied UDP-sugar nucleotides (15).

Based on these, they concluded that spHasA and DG42 proteins were hyaluronan synthases themselves capable of transferring both UDP-GlcNAc and UDP-GlcA. Recently, Tlapak-
Simmons et al. (16) have demonstrated that the functional sizes of the two streptococcal hyaluronan synthases were monomers by the radiation inactivation analysis and matrix-assisted laser desorption ionization/time of flight mass spectrometry, proposing that the active streptococcal hyaluronan synthases are monomers. However, it is still controversial whether these are also the case for the mammalian systems. In order to make it clear, it would be necessary to examine the hyaluronan synthetic activity of the pure recombinant mammalian HAS protein in vitro and to characterize the domain structure responsible for each glycosyltransferase activity.

In this study, we first demonstrated that mouse HAS1 gene product expressed in an in vitro translation system as well as the purified single mouse HAS1 protein had the ability to synthesize HA with exogenously supplied UDP-GlcA and UDP-GlcNAc. Based on this result, we then characterized both the GlcNAc and GlcA transferase activities of this protein by using site-directed mutagenesis of the HAS1 gene, which in these assay systems has enabled us to specify amino acid residues for those activities, and found that within the central loops domain several amino acid residues were responsible for the GlcNAc transferase activity and another amino acid residue responsible for the GlcA transferase activity, respectively.

**Experimental procedures**

**Materials**—UDP-[14C]GlcA and UDP-[3H]GlcNAc were purchased from NEN Life Science Products. [35S]Methionine was from ICN Pharmaceuticals, Inc. UDP-GlcA, UDP-GlcNAc, bovine cardiofilin, and *Streptomyces* chitinase were from Sigma. The pFLAG-CMV-2 expression vector, anti-FLAG M2 affinity gel, and FLAG-tagged bacterial alkaline phosphatase (FLAG-BAP) protein were from Eastman Kodak Co. Dulbecco's modified Eagle's medium (DMEM) and the methionine-depleted DMEM were from Life Technologies, Inc. Fetal bovine serum was from Cytosystems Pty. Ltd. Pfu DNA polymerase was from Stratagene. Gene Pulser transfection apparatus and Bio-Rad protein assay kit were from Bio-Rad. ECL detection system was from Amersham Pharmacia Biotech. Single tube protein system 2 and pCITE-4c(+) vector were from Novagen. DNA sequence ABI 310 was from Applied Biosystems, Foster City, CA. HiLoad 16/60 Superdex 30 pg and PD-10 column were from Amersham Pharmacia Biotech, Uppsala, Sweden. Pronase was from Roche Molecular Biochemicals, Mannheim, Germany. Mutan®-Express Km kit was from TaKaRa, Tokyo, Japan. CHAPS was from Dojindo Laboratories, Kumamoto, Japan. Fuji BAS-2000 II system was from Fuji Photo Film Co., Ltd, Tokyo, Japan. *Streptomyces* hyaluronidase, chito-oligosaccharide, and chitohexaose were from Seikagaku Corp., Tokyo, Japan. Standard HA polysaccharides were from Seikagaku Corp., Tokyo, Japan. Standard HD polysaccharides of 2.5, 3.5, and 4.5 kDa were gifts of Dr. Youji Ohnuki, Tokyo Research Institute for Medical Science, Tokyo, Japan.

**Construction and Transfection of FLAG Epitope-tagged HAS1 Expression Vector**—A mouse HAS1 polymerase chain reaction fragment was amplified through 20 cycles using Pfu DNA polymerase and the following primers: forward, 5'-GATAGATCTGAGACAGGACATGC-3' (this primer contains a BsgII site and corresponds to amino acids 282-307) and reverse, 5'-CAGCCGCCTCGCGTTGTCCCCAGC-3' (corresponds to amino acids 924-943). The resulting polymerase chain reaction fragment, which included a BspHI site, was excised from the BsgII and BspHI sites and gel-purified. A 3'-fragment excised from a full-length HAS1 cDNA at BspHI and BsgII sites was also gel-purified. Two of these two HAS1 fragments were subcloned into the BsgII site of pFLAG-CMV-2 vector to create a pFLAG-HAS1 construct. Sequences of the resultant constructs were determined by DNA sequencing using an automated DNA sequencer ABI 310.

The FLAG-tagged vector was transfected into COS-1 cells by electroporation according to the manufacturer's instructions using the Gene Pulser transfection apparatus. The transfected COS-1 cells were cultured for 3 days in DMEM containing 10% (w/v) fetal bovine serum and 2 mM l-glutamine at 37 °C. For control cells were transfected with the pFLAG-CMV-2 vector.

**Hyaluronan Synthase Assay**—HA synthase activity was monitored using UDP-[14C]GlcA (272.5 mCi/mmol) or UDP-[3H]GlcNAc (37 mCi/mmol) as described previously (7). Briefly, the HAS transfectedants were washed, harvested, and disrupted by sonication in 10 mM Hepes-NaOH, pH 7.1, 0.5 mM dithiothreitol containing 0.25 mM sucrose. A suspension of the disrupted cells was centrifuged in a Beckman TLS rotor at 43,000 rpm for 60 min to give a high speed pellet. A pellet prepared from the transfectedants was suspended as the membrane fraction in 0.1 ml of the reaction buffer containing 50 mM Tris-HCl, pH 7.1, 5 mM dithiothreitol, 15 mM MgCl₂, 2 mM tetradsodium pyrophosphate, 1.0 mM UDP-GlcNAc, 0.1 mM UDP-GlcA, and either 2.0 Ci of UDP-[14C]GlcA or 5.0 μCi of UDP-[3H]GlcNAc for the incorporation of [14C]GlcA, or 5.0 μCi of UDP-[3H]GlcNAc for the incorporation of [3H]GlcNAc, which gave the approximately equal specific activity to both the substrates. After the incubation for 0.5–2.0 h at 37 °C, the reaction was stopped by boiling, and the mixture was further incubated with or without 1% (w/v) SDS, 14C- or 3H-labeled polysaccharides were separated by chromatography on HiLoad 16/60 Superdex 30 pg (16 × 60 cm) and eluted with 0.2 M ammonium acetate, and 1-ml fractions were collected for determination of the radioactivity. Radioactivity incorporated into hyaluronan polymer was calculated from the *Streptomyces* hyaluronidase-sensitive radioactivity.

**Purification of FLAG-tagged HAS1 Fusion Protein**—Membrane fraction containing the expressed fusion protein was suspended in Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) with 0.2% (w/v) CHAPS and mixed well. After the centrifugation at 10,000 rpm for 30 min at 4 °C, the supernatant solution was obtained as the detergent extract. A affinity fraction obtained from the detergent extract by anti-FLAG M2 affinity chromatography according to the method recommended by the manufacturer. Then, the affinity fraction was subjected to SDS-PAGE (8% (w/v) gel) under non-reducing conditions, and after the electrophoresis the gel was washed two times with 50 mM Tris-HCl, pH 7.1, in the presence of 0.5% (w/v) CHAPS, followed by two washes with 50 mM Tris-HCl, pH 7.1. The gel was cut out at intervals of 1 mm length from the position of the green marker (97 kDa) of the Kaleidoscope-prestained standards toward the bottom front to give 10 fractions, and each gel was homogenized in 50 mM Tris-HCl, pH 7.1, at 4 °C to solubilize proteins. After the centrifugation at 10,000 rpm for 5 min, the supernatant solutions were obtained as the SDS-PAGE fractions. FLAG affinity fraction and SDS-PAGE fraction without storage were incubated in 0.1 ml of the reaction mixture containing 50 mM Tris-HCl, pH 7.1, 5 mM dithiothreitol, 15 mM MgCl₂, 2 mM tetradsodium pyrophosphate, 1 mM UDP-GlcNAc, 0.1 mM UDP-GlcA, and 2.5 μCi of UDP-[14C]GlcA at 37 °C from 0.5 to 2.0 h, and their HAS activities were measured by the method described above.

**Recombinant Hyaluronan Synthase Assay**—FLAG affinity fraction and/or SDS-PAGE fraction were mixed in 0.1 ml of the reaction mixture containing 50 mM Tris-HCl, pH 7.1, 5 mM dithiothreitol, 15 mM MgCl₂, 2 mM tetradsodium pyrophosphate, 1 mM UDP-GlcNAc, 0.1 mM UDP-GlcA, and 2.5 μCi of UDP-[14C]GlcA at 37 °C in the same buffer solution (50 mM Tris-HCl, pH 7.1, 5 mM dithiothreitol, 15 mM MgCl₂, and 2 mM tetradsodium pyrophosphate) without mixing. The two-layer fractions were incubated for 0.5–2.0 h without mixing, and the upper layers of the reaction mixtures were incubated at 37 °C to stop reaction. HAS activities were measured by the method described above. In several preliminary tests, the lower layer of 0.7 μM CHAPS solution was diluted with 10 volumes of the buffer solution and applied onto the PD-10 columns to remove CHAPS. Radioactive hyaluronan if present in the eluates was measured as described above. No significant radioactivity was detected in any case, probably due to the incapability for hyaluronan to diffuse into the CHAPS solution with such a highly viscous property. Therefore, only the upper layers of the reaction mixtures were subjected to the measurements of HAS activities otherwise noted.

**Assay for Chito-oligosaccharide Synthetic Activity**—The membrane fraction was incubated in 0.1 ml of the reaction buffer containing 50 mM Tris-HCl, pH 7.1, 5 mM dithiothreitol, 15 mM MgCl₂, 2 mM tetradsodium pyrophosphate, 1.0 mM UDP-GlcNAc, and 10.0 μCi of UDP-[3H]GlcNAc at 37 °C for 2.0 h. The reaction was stopped by adding 100 ml of water and subsequent boiling for 2 min, and the products were subjected to the assay for chito-oligosaccharide synthetic activity as described previously (17) with a minor modification. After the centrifugation, half of the supernatant solution was treated with 200 μg/ml Pronase at 37 °C for 5 h. The other half of the original supernatant solution was treated with 100 μg/ml chitinase as described in the unit of *Serratia* chitinase at 25 °C overnight before the Pronase treatment. These samples were applied onto the PD-10 columns to remove the fractions containing the labeled substrate and its degradation products. After concentration, they were subjected to chromatography on HiLoad 16/60 Superdex 30 pg as described above. Chito-oligosaccharide synthetic activity was determined by the *Serratia* chitinase-sensitive radioactivity.

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dioactivity in the labeled polysaccharides. As control, the isolated membrane fraction was also incubated in the reaction buffer in the presence of 0.1 mM UDP-GlcA and 2.5 μCi of [14C]GlcA as a donor substrate at 37°C for 4 h, and samples were analyzed as described above.

**HA and Chito-oligosaccharide Synthetic Activities of Mutant HAS1 Protein**—The purified HAS1 protein was generated by in vitro site-directed mutagenesis using Mutan®-Express Km system according to the method described by the manufacturer. All mutations were confirmed by sequencing the DNAs. All clones were ligated into pFLAG-CMV-2 expression vector as described above and transfected into COS-1 cells. HA and chito-oligosaccharide synthetic activities of these mutants were compared after the incubation at 37°C for 2 h with the membrane fractions containing 3.0 mg of total protein as described above. Expression levels of all mutants and wild type fusion proteins were monitored by their staining intensities on Western blotting with anti-FLAG M5 antibody as described above. Protein contents of the membrane fractions were determined using the Bio-Rad protein assay kit.

In Vitro Transcription/Translation of FLAG-tagged HAS1 Fusion Protein—pFLAG-HAS1 was used for in vitro transcription/translation with the single tube protein system 2 according to the protocol of the manufacturer for the comparison of the migration ratio with the purified HAS1 protein on Western blotting. To examine the hyaluronan synthase activity of the protein expressed in an in vitro transcription/translation system, pCITE-FLAG-HAS1 expression vector was constructed. pFLAG-HAS1 was cut with SacI, blunted, and digested with EcoRV. After the fragment was gel-purified, it was subcloned into the blunting site of pCITE-4c(+) vector. After transcription/translation reaction, the solution was incubated in the reaction mixture as described above in the presence of 1.0 mM UDP-GlcNAc, 0.1 mM UDP-GlcA, and 2.5 μCi of [14C]GlcA under the reconstitutive conditions described above. The incubation was also carried out without subjecting to the reconstitutive conditions. HAS activity was measured as described above.

 Autoradiogram of SDS-PAGE of FLAG Affinity Fraction—Cells were cultured for 60 h after the transfection. After three washes with methionine-depleted DMEM, the cells were incubated in the methionine-depleted DMEM containing dialyzed 10% fetal bovine serum and 0.1 mM cysteine for 12 h. The crude membrane fractions were prepared from the [35S]methionine-labeled cells as described above. The labeled FLAG affinity fraction was obtained and subjected to SDS-PAGE (8% (w/v) gel) under the non-reducing condition as described above. The labeled protein was visualized by bio-imaging analyzer (Fuji BAS-2000 II system).

 Western Blotting of FLAG-tagged HAS1 Fusion Protein and Their Mutant Proteins—Membrane fraction, FLAG affinity fraction, and SDS-PAGE fraction were completely solubilized in the sample buffer (50 mM Tris-Cl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, with or without 6% 1-mercaptoethanol) and were subjected to SDS-PAGE (8% (w/v) gel), transferred to nitrocellulose membranes. Staining was performed according to the manufacturer’s instructions using anti-FLAG peptide antibody M5, followed by peroxidase-conjugated anti-mouse IgG antibody. The immune complexes were detected by the ECL detection system. The presence of single recombinant protein band (Fig. 3A) was confirmed by sequencing the DNAs. All clones were ligated into pFLAG-CMV-2 and COS-1 cells were transfected with this construct or with the vector alone as a control. The membrane fractions were prepared and assayed for the HAS activity. The fraction prepared from the cells transfected with pFLAG-HAS1 synthesized polysaccharide sensitive to Streptomyces hyaluronidase in contrast to no virtual synthesis of the polysaccharide by the control (Fig. 1). The polysaccharide was HA, and consisted of at least more than 24 residues of monosaccharide since it was eluted in the void volume of HiLoad 16/60 Superdex column. The presence of both UDP-GlcA and UDP-GlcNAc was required for the synthesis of HA. The ha lactase and CHAPS were seen in the membrane fractions of the pFLAG-HAS1-transfected cells, and subjected to the anti-FLAG M2 antibody column. Significant hyaluronan synthetic activity was recovered in the FLAG affinity fraction, although recovery of the total activity was quite low (about 4% of that of the membrane fraction) (Table I). To assess the purity of HAS1 protein, the affinity fraction labeled with [35S]methionine was then subjected to SDS-PAGE (8% (w/v) gel) under the non-reducing conditions. Autoradiography of the sample obtained from the pFLAG-HAS1-transfectant revealed the presence of four major protein bands between 87 and 44.1 kDa (Fig. 2A). Only a 60-kDa band of the protein, which was not observed in the control sample, reacted with anti-FLAG M5 antibody on Western blotting (Fig. 2B), suggesting that this band was a fusion protein. The other three major proteins appeared to be membrane proteins not related to HAS1 protein because they were also seen in the control sample and showed no reactivity with anti-FLAG M2 antibody. To further examine whether or not this single protein band of 60 kDa had the HAS activity, we prepared the non-labeled affinity fraction in a large scale and subjected to SDS-PAGE (8% (w/v) gel) under non-reducing conditions. Ten SDS-PAGE fractions were obtained by cutting out the gel around the single protein band at 1-mm intervals (Fig. 2A). Protein from each fraction was solubilized by homogenizing the gel slices and subsequent removal of the gel debris by centrifugation and incubated in the reaction buffer containing both sugar nucleotide precursors. The HAS activity was only recovered in the fraction containing a 60-kDa protein band (Fig. 3A), and synthesized HA was eluted in the void volume (Fig. 3B). The presence of single recombinant fusion protein in this fraction was confirmed by silver staining and by the reactivity with anti-FLAG M5 antibody on Western
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**TABLE I**

Purification of HAS1 protein and examination of the activity of hyaluronan synthesis

COS-1 cells were transfected as described under "Experimental Procedures" with a plasmid containing the mouse HAS1 cDNA (pFLAG-HAS1). HAS activities in the membrane fraction, FLAG affinity fraction, and SDS-PAGE fraction were determined as described under "Experimental Procedures." Values represent means ± S.D. of triplicate cultures.

| Fractions          | CHAPSa | Total activityb | Specific HAS activityc | Purification | Recovery |
|--------------------|--------|-----------------|------------------------|--------------|----------|
|                    |        | dpm/h           | pmol h/ug              | f.d.d        | %        |
| Membrane fraction  |        |                 |                        |              |          |
| Control mock transfectant | +     | 30 ± 3          | 0.01                   | 1            | 100      |
| pFLAG-HAS1 transfectant | +     | 14,932 ± 1,179  | 1.57 ± 0.12            | 1            | 100      |
| FLAG affinity fraction | +     | 15,015 ± 1,223  | 1.57 ± 0.13            | 1            | 100      |
| SDS-PAGE fraction  |        |                 |                        |              |          |
| −                  | 596 ± 54 | 62.6 ± 5.7        | 40                     | 4.0          |
| +                  | 1486 ± 143 | 156.0 ± 15.0    | 99                     | 9.9          |

*a HA synthesize activity was measured under the reconstitutive conditions.

*b HA synthesize activity, radioactivity of [14C]glucuronic acid incorporated into HAb. The values given are means ± S.D. for three experiments.

*c Specific activity, picomoles of glucuronic acid incorporated into HAb/µg of total protein. Total protein in SDS-PAGE fraction was calculated from the amount of the FLAG-tagged HAS1 protein in this fraction on the assumption that this fraction only contained the FLAG-tagged HAS1 protein. The values given are means ± S.D. for three experiments.

the total amount of HAS1 protein (37%), which suggested that the solubilization of HAS1 protein with the detergent from the membrane markedly reduced the HAS enzyme activity. We therefore examined the effects of the detergent solubilization on the enzyme properties by comparing the kinetic properties of HAS1 proteins before and after the solubilization and purification, which were analyzed by measuring the substrate-dependent changes in the incorporation of radiolabeled precursor sugar nucleotides into HA (Fig. 4). The increased concentrations of either substrate gave a V_max saturation profile of HAS1 activity of the membrane fraction. In contrast to this, the kinetic behaviors of the HAS1 activities of both the FLAG affinity fraction and the SDS-PAGE fraction were quite different. The lowest concentration of either substrate (1 µM) that we tested gave the highest HAS1 activity, and the increased concentration of either substrate from 1.0 to 1000 µM, where both substrates were at the same concentrations brought about the rapid decrease in the activity (Fig. 4). Recent reports (16, 23, 24) should be referred to here, describing that cardiolipin, one of membrane phospholipid components, is required for the maximum activity and stability of the purified streptococcal hyaluronan synthases. We, therefore, examined whether depletion of some membranous factors by the solubilization may have caused reduction of the activity and change in the enzymatic properties and those factors may enhance the HAS activity. Contrary to the reported effect on streptococcal hyaluronan synthases, no significant enhancement of the HAS1 activity was observed with either the FLAG affinity fraction or the SDS-PAGE fraction when cardiolipin was added into the reaction mixture at the final concentrations of 1.0–2.0 mM as described in the above reports (data not shown). However, interestingly, when the reaction was performed by incubating the reaction mixture containing the purified HAS1 protein, 1 mM UDP-GlcNAc, 0.1 mM UDP-GlcA, and 2.5 µCi of [14C]GlcA in the buffer solution of 50 mM Tris-HCl, pH 7.1, 5 mM dithiothreitol, 15 mM MgCl₂, and 2 mM tetrasodium pyrophosphate, layered onto 0.7 M CHAPS in the same buffer solution without mixing, which we named the reconstitutive assay conditions hereinafter, the kinetic behaviors of the detergent-solubilized and purified HAS1 protein of either the SDS-PAGE fraction or the FLAG affinity fraction became almost similar to those of the membrane-bound HAS1 protein fraction in that the increased concentration of either substrate gave a V_max saturation profile of HAS1 activity (Fig. 4). In addition, under the reconstitutive conditions, the K_M value for each substrate of the SDS-PAGE fraction was also at the same level as that for the membrane fraction although the V_max per microgram of HAS1
activity in the membrane fraction, which suggests us further consideration on the methods for the solubilization and purification of HAS1 protein. However, based on the specific activity of the SDS-PAGE fraction, the detergent solubilization from the membrane fraction and subsequent purification gave about 650-fold purification of the HAS1 protein (Table I).

**HA Synthesis by in Vitro Translated HAS1 Protein**—In order to further exclude the possibility that any other associated components covalently bound to purified HAS1 protein in the SDS-PAGE fraction, SDS-PAGE and Western blotting of the fraction were performed under the reducing conditions and then the pattern was compared with that of the HAS1 gene product expressed in an *in vitro* transcription/translation system. Both the proteins that are expressed *in vivo* by cells and translated in an *in vitro* system showed a single band migrating at the same position on SDS-PAGE (Fig. 3C, lane 4), suggesting that no other components covalently bound to a fusion protein expressed in COS-1 cells and, therefore, that HAS1 protein alone without any other components has the capacity to exhibit HAS activity. However, there might be still a possibility that HA was synthesized additionally from the short hyaluronan oligosaccharide covalently bound to the HAS1 protein that could not be recognized from the migration patterns on SDS-PAGE. To assess this possibility and also to obtain further evidence to show that HAS1 protein alone has the capacity to synthesize HA, we examined the HAS activity of the gene product expressed in an *in vitro* transcription/translation system. When the reaction mixture was subjected to the reconstitutive assay conditions where the mixture was layered onto the buffer solution containing 0.7 M CHAPS without mixing, the polymer that was eluted in the void volume of the HiLoad 16/60 Superdex column and sensitive to *Streptomyces* hyaluronidase was synthesized. However, no significant activity was found without subjecting the reaction mixture to the reconstitutive reaction conditions (data not shown). Control sample expressed only the pCITE-4c(+) vector showed no activity to synthesize the polymer or short oligosaccharide, even under the reconstitutive conditions (data not shown).

**Chito-oligosaccharide Synthetic Activity of HAS1 Protein**—The above results suggest that a single protein has the capacity to synthesize hyaluronan by catalyzing alternative transfer of GlcNAc residue from UDP-GlcNAc and GlcA residue from UDP-GlcA, respectively. Therefore, one can expect that two different catalytic sites on the one polypeptide that should have specific and different amino acid motifs or residues are responsible for each glycosyltransferase activity. Determination of these sites may be useful to further study the mechanism and regulation of hyaluronan synthesis. For it, it is necessary to develop the assay systems to detect each transferase activity of GlcNAc to the GlcA residue or GlcA to the GlcNAc residue. Semino et al. (17) reported previously that *X. laevis* DG42 protein obtained in an *in vitro* transcription/translation system synthesized the array of chito-oligosaccharides that are composed of GlcNAc with β(1→4) linkage. Based on this observation, we assumed that HAS1 protein has the activity of chito-oligosaccharide synthesis under certain conditions, because the transferase activity of GlcNAc residue is required for the HA synthesis. The membrane fraction was incubated in a large scale under several different conditions such as the presence of only one donor substrate or the exposure to the lower pH condition, and the products were analyzed by chromatography on HiLoad 16/60 Superdex 30 pg. When incubated with UDP-GlcNAc alone, the product was eluted in the Superdex fractions where oligosaccharides were eluted. The oligosaccharide thus obtained was sensitive to *Serratia* chitinase, but not to *Streptomyces* hyaluronidase (Fig. 5). In addition, the product was not

**protein in the SDS-PAGE fraction was still lower than that of the membrane fraction (Table II). The results suggested that the detergent solubilization and subsequent purification of HAS1 protein reduced the HAS enzyme activity with change in the kinetic properties of the enzyme.**

**Under the reconstitutive assay conditions, the total activity of the FLAG affinity fraction was about 10% of the membrane fraction (Table I). Considering that the recovery of HAS1 protein in the FLAG affinity fraction was estimated to be about 30% of that in the membrane fraction, the detergent solubilization and subsequent affinity purification caused the loss of about 70% of HAS1 protein and of about 90% of the HAS**
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**Fig. 4. Kinetic profiles of HAS1 protein in the membrane fraction and in the SDS-PAGE fraction with respect to UDP-GlcNAc or UDP-GlcA concentrations, and the effects of the reconstitutive assay conditions.** HAS activities of HAS1 protein in the membrane fraction (○) and in the SDS-PAGE fraction (□) were determined at various concentrations of UDP-GlcNAc (A) or UDP-GlcA (B) while holding the concentration of the radiolabeled another substrate at 1.0 mM as described under “Experimental Procedures.” The profiles were different between the membrane fraction and the SDS-PAGE fraction, and HAS activities of the purified HAS1 protein were markedly reduced at the higher concentrations of either of the two substrates. Under the reconstitutive assay conditions (the reaction in the mixture overlaying the buffer containing 0.7 M CHAPS), HAS activities of HAS1 protein in the SDS-PAGE fraction (●) were determined. The kinetic profiles of the purified HAS1 protein with respect to UDP-GlcNAc (○) or UDP-GlcA (□) concentrations under these conditions became similar to those of the membrane fraction under the normal conditions. The kinetic profiles of HAS1 protein in the membrane fraction under the reconstitutive assay conditions were almost identical to the ones under the normal assay conditions (data not shown). In addition, the profiles of HAS1 protein in the FLAG affinity fraction under the normal and reconstitutive conditions were essentially identical to the ones in the SDS-PAGE fraction (data not shown). The amounts of the HAS1 protein in the membrane and the SDS-PAGE fractions were calculated by measuring the staining intensities of FLAG-tagged HAS1 protein on the membranes using anti-FLAG peptide antibody and known amounts of FLAG-tagged BAP protein as standards as described under “Experimental Procedures.”

**TABLE II**

| Purified material | $K_m$ for UDP-GlcNAc (μM) | $V_{max}$ (pmol/min/μg) | $K_m$ for UDP-GlcA (μM) | $V_{max}$ (pmol/min/μg) |
|-------------------|---------------------------|------------------------|-------------------------|------------------------|
| Membrane-bound    | 950 ± 70                   | 51.5 ± 4.5             | 60 ± 5                  | 46.2 ± 4.1             |
| Detergent-solubilized | 870 ± 60                  | 24.2 ± 2.1             | 55 ± 5                  | 21.3 ± 1.9             |

* Picomoles of glucuronic acid or N-acetyl glucosamine incorporated into HA/min/μg of HAS1 protein.

bound to the DEAE-Sepharcel column (data not shown). These results suggested that it was chito-oligosaccharide. Molecular mass of this oligosaccharide was calculated to be less than 3.5 kDa. The control membrane fraction did not yield any radiolabeled oligosaccharides. The activity of chito-oligosaccharide synthesis per a HAS1 protein basis was 18–22% of that of hyaluronan synthesis at the concentrations of UDP-GlcNAc (0.1 mM) and UDP-GlcA (0.05, 0.2, or 1.0 mM). Since it is generally accepted that mammalian cells have no activity to synthesize chitin or chitin oligosaccharides, the synthetic activity of chito-oligosaccharide under such an abnormal condition is likely to represent the GlcNAc transferase activity for hyaluronan synthesis. Hereinafter, we used this activity for the assay of this transferase activity.

In contrast to the synthetic activity of chito-oligosaccharide, when the incubation was carried out under the condition where UDP-GlcA alone was present as a donor substrate, the membrane fraction did not yield any radiolabeled polysaccharides or oligosaccharides in the reaction mixture (Fig. 5).

**Identification of Amino Acid Residues Responsible for GlcNAc and GlcA Transferase Activities—** The mammalian HAS gene products consist of three domains, and the catalytic activity of HA synthase is estimated to be in the central cytoplasmic loop domain of all HAS proteins (19). This motif is also conserved in the NodC proteins, which synthesize chito-oligosaccharides, and in cellulose synthases, which catalyze glucosyl β-1→4 linkage formation. These findings suggest that this motif may be responsible for the transferase activity of GlcNAc to GlcA with a β-1→4 linkage in HA synthesis and for the synthesis of GlcNAc oligosaccharide with a β-1→4 linkage under the conditions described above. Amino acid residues consisting of this motif and their location on the HAS1 protein are Asp242, Asp344, Glu380, Arg383, and Trp384 (Fig. 6).

It is of note that there are other amino acid sequence alignments in the cytoplasmic central loop domains of all HAS proteins that were also conserved among rat, mouse, rabbit, and of human UDP-glucuronosyltransferases (UDP-GTs), although the direction of the amino acid sequence order of the UDP-GTs is reversed. These UDP-GTs catalyze the glucuronidation of potentially toxic endogenous compounds such as catecholamines, bilirubin as well as environmental chemicals. Amino acid residues consisting of this alignment and their location on HAS1 protein are Ser311, Gly312, Pro313, and Leu314 (Fig. 7). It has been shown that these residues form a cluster in the C-terminal conserved domains of UDP-GTs and are localized in the region that has the activity to bind UDP-GlcA and corresponds to the 2B4 peptide (299–446) of human liver UDP-GT (20, 21). Therefore, it is possible that these residues may be responsible for the GlcA transferase activity of all HAS proteins. All residues are localized between Asp242 and Asp344.
of HAS1 protein, which are the predicted residues consisting of the motif involved in the GlcNAc transferase activity as described above.

Based upon these predictions, we created a series of mutant HAS1 proteins in which these residues are substituted to their analogues by means of site-directed mutagenesis and analyzed the effects on both HA and chito-oligosaccharide synthetic activities. Mutation was introduced to have one amino acid residue substitution with its analogue, and both the synthetic activities of the membrane fractions were examined as described under “Experimental Procedures” (Table III). Substitution of Asp242 with glutamic acid, Asp344 with glutamic acid, or Trp384 with tyrosine caused severe loss of both HA and chito-oligosaccharide synthetic activities. Interestingly, substitution of Leu314 with valine did not affect the activity of chito-oligosaccharide synthesis, while it also caused severe loss of HA synthase activity. Substitution of Gln380 with asparagine or Arg383 with lysine left 10–15% of both HA and chito-oligosaccharide synthetic activities. Substitution of Leu314 with isoleucine again caused significant decrease of HA synthase activity but not chito-oligosaccharide synthetic activity. There were no significant effects on both the activities of substitution of Ser311 with asparagine, Gly 312 with proline, or Pro 313 with glycine. The mutant HAS1 proteins exhibited similar $K_m$ values for the substrates to those of the wild type HAS1 protein with the exception of D344E, whose activities were too low to determine the $K_m$ values (Table III). In addition, no polysaccharide synthesis was detected when the membrane fraction from the pFLAG-HAS1 transfectant was incubated with UDP-14C(GlcA alone as a donor substrate). $V_o$, excluded volume, 2.5- and 3.5-kDa HA; $V_c$, volume of total column, 120 ml. No polysaccharide synthesis was detected when the membrane fraction from the pFLAG-HAS1 transfectant was incubated with UDP-14C(GlcA alone as a donor substrate). $V_o$, excluded volume, 2.5- and 3.5-kDa HA; $V_c$, volume of total column, 120 ml. No polysaccharide synthesis was detected when the membrane fraction from the pFLAG-HAS1 transfectant was incubated with UDP-14C(GlcA alone as a donor substrate). $V_o$, excluded volume, 2.5- and 3.5-kDa HA; $V_c$, volume of total column, 120 ml. No polysaccharide synthesis was detected when the membrane fraction from the pFLAG-HAS1 transfectant was incubated with UDP-14C(GlcA alone as a donor substrate). $V_o$, excluded volume, 2.5- and 3.5-kDa HA; $V_c$, volume of total column, 120 ml. No polysaccharide synthesis was detected when the membrane fraction from the pFLAG-HAS1 transfectant was incubated with UDP-14C(GlcA alone as a donor substrate). $V_o$, excluded volume, 2.5- and 3.5-kDa HA; $V_c$, volume of total column, 120 ml. No polysaccharide synthesis was detected when the membrane fraction from the pFLAG-HAS1 transfectant was incubated with UDP-14C(GlcA alone as a donor substrate). $V_o$, excluded volume, 2.5- and 3.5-kDa HA; $V_c$, volume of total column, 120 ml. No polysaccharide synthesis was detected when the membrane fraction from the pFLAG-HAS1 transfectant was incubated with UDP-14C(GlcA alone as a donor substrate). $V_o$, excluded volume, 2.5- and 3.5-kDa HA; $V_c$, volume of total column, 120 ml. No polysaccharide synthesis was detected when the membrane fraction from the pFLAG-HAS1 transfectant was incubated with UDP-14C(GlcA alone as a donor substrate). $V_o$, excluded volume, 2.5- and 3.5-kDa HA; $V_c$, volume of total column, 120 ml. No polysaccharide synthesis was detected when the membrane fraction from the pFLAG-HAS1 transfectant was incubated with UDP-14C(GlcA alone as a donor substrate). $V_o$, excluded volume, 2.5- and 3.5-kDa HA; $V_c$, volume of total column, 120 ml. No polysaccharide synthesis was detected when the membrane fraction from the pFLAG-HAS1 transfectant was incubated with UDP-14C(GlcA alone as a donor substrate). $V_o$, excluded volume, 2.5- and 3.5-kDa HA; $V_c$, volume of total column, 120 ml.
**Ha Synthesis but not for Chito-Oligosaccharide Synthetic Activities**

Amino acid residues which were predicted to be responsible for glycosyltransferase activities were substituted by their analogues. The effects on HA and chito-oligosaccharide synthetic activities and $K_m$ constants were determined using the membrane fractions as described under "Experimental Procedures."

| Substitutions | Relative synthetic activity % | $K_m$ for UDP-GlcNAc | $K_m$ for UDP-GlcA |
|---------------|-----------------------------|----------------------|-------------------|
| Wild type     | 100.00                      | 100.00               | 0.95              | 0.06             |
| D242E         | 1.00                        | 0.9                  | 1.10              | 0.05             |
| D344E         | <0.01                       | <0.01                | ND$^a$            | ND$^a$           |
| Q380N         | 12.40                       | 11.30                | 0.90              | 0.04             |
| R359R         | 15.20                       | 13.90                | 1.05              | 0.06             |
| W384Y         | 1.30                        | 1.05                 | 1.20              | 0.07             |
| S311N         | 99.10                       | 98.70                | NT$^b$            | NT$^b$           |
| G312P         | 100.30                      | 99.10                | NT$^b$            | NT$^b$           |
| P313G         | 98.70                       | 99.40                | NT$^b$            | NT$^b$           |
| L314I         | 24.70                       | 98.60                | NT$^b$            | NT$^b$           |
| L314V         | 1.05                        | 98.50                | 0.97              | 0.06             |

$^a$ ND, not determined.

$^b$ NT, not tested.

The time courses of the reactions by those mutant HAS1 proteins (the reaction rates at the 0.5-, 1-, and 2-h incubations) exhibited the similar profiles (data not shown), suggesting that the loss or decrease in the activity caused by the mutation was not due to the alteration of the substrate binding affinity and/or the instability or denaturation of those mutant proteins under the assay conditions. Furthermore, the amounts of a series of mutant proteins expressed were almost the same as that of the wild type HAS1 protein (Fig. 8). These results suggested that Asp$^{242}$, Asp$^{344}$, and Trp$^{384}$ are essential for both HA and chito-oligosaccharide synthetic activities, and Leu$^{144}$ is essential for HA synthesis but not for chito-oligosaccharide synthetic activity.

**DISCUSSION**

The present study was undertaken whether mammalian HAS1 protein alone was enough to synthesize HA. Detergent-solubilized, purified HAS1 protein exhibited the HA activity and similar kinetic profiles to those of the membrane-bound form under the reconstitutive assay conditions. Moreover, the same gene product expressed in an active form under the reconstitutive assay conditions. The maximal activity was rather observed at the lowest concentration tested of UDP-GlcNAc or UDP-GlcA, even when the concentration of the other substrate was high. However, under the reconstitutive assay conditions, the detergent-solubilized and purified HAS1 proteins showed similar kinetic profiles to the membrane-bound form. The suitable conformation for HAS1 protein to express the activity may be reconstituted and stabilized in the reaction mixture layered onto the high concentration CHAPS. In the absence of such conditions, the activity of HAS 1 protein might be greatly influenced by the concentration of substrate molecules in that the lower concentrations of the substrates abruptly activate the enzyme while the higher concentrations of the substrates rather suppress the activity. The reconstitutive assay conditions appeared to mimic the reaction in the membranes, although it is difficult to assess how the purified HAS1 protein corrected the natural conformation. However, under the reconstitutive conditions hyaluronan might be synthesized by the proteins in the boundary layer between the reaction mixture and the CHAPS solution, since no activation of the HA activity was observed when the reaction mixture was mixed and homogenized with the CHAPS solution. Hyaluronan synthesized under the reconstitutive assay conditions could be diffused into the upper layer of the reaction solution because no detectable amount of hyaluronan was recovered in the lower layer of the CHAPS solution.

Cardiolipin was found to be effective for the activation and stability of the detergent-solubilized and purified streptococcal HAS proteins (24). However, this was not the case for mammalian HAS1 proteins, at least judging from our experiment where we examined the activity in the presence of bovine cardiolipin at the concentrations of 1.0–2.0 mM. Considering the fact that the amount of this molecule in the mammalian plasma membrane is lower than that in the plasma membrane of prokaryote, cardiolipin may not be involved in the enhancement of HAS activity in mammals.

The present results showed that HAS1 protein had the ac-
tivity to synthesize chito-oligosaccharide when UDP-GlcNAc alone was present as a donor substrate and demonstrated that the amino acid residue, Leu<sup>214</sup>, on the protein was essential for HA synthesis but not chito-oligosaccharide synthetic activity, suggesting that the enzyme is bifunctional and the catalytic sites responsible for hyaluronan synthesis may include the one for chito-oligosaccharide synthesis. Regarding the function of DG42 protein, there are two contradictory reports suggesting that this protein may be bifunctional. Semino and Robins (17, 25) showed that DG42 protein synthesized chito-oligosaccharide both in an in vitro transcription/translation system and by overexpression in mouse 3T3 cells. On the other hand, Meyer and Kreil (26) reported that DG42 protein synthesized HA in both cells and the membrane fractions when the DG42 cDNA was expressed in rabbit kidney cells (RK13) and also in human osteosarcoma (tk<sup>-</sup>) cells. One possible interpretation to explain this discrepancy may be related to our present findings that HAS1 protein is able to catalyze the synthesis of either chito-oligosaccharide or hyaluronan, depending on the supplies of two donor substrates (27). For example, the concentration of UDP-GlcA may affect the catalytic function of HAS1 protein at least in vitro. Semino and Robbins (25) have discussed the possibility that chito-oligosaccharide may serve as a primer for HA synthesis, based on their additional finding that HA synthesis was inhibited by the presence of chitinase in the reaction buffer. We examined the effect of the presence of <i>Serratia</i> chitinase on HA synthesis by recombinant HAS1 protein, and at least under our conditions we neither found formation of the oligosaccharide sensitive to <i>Serratia</i> chitinase even at the early period of the incubation in the presence of both UDP-GlcNAc and UDP-GlcA nor detected the inhibition of HA synthesis by the addition of chitinase (data not shown). Our results weaken the possibility of priming function of the chito-oligosaccharide.

All HAS proteins have the amino acid motif consisting of D,D,QXXRW, which is essential for the glycosyltransferase activity with β1→4 linkage. The motif has been suggested by Nagahashi et al. (19), who successfully determined amino acid residues essential for the catalytic activity of chitinase 2. This motif is also conserved in other known glycosyltransferases, which catalyze the synthesis of oligosaccharides with β1→4 linkage formation such as NosC proteins and cellulose synthases. The present results confirmed that this motif is also essential for the synthesis of hyaluronan in HAS1 protein. Moreover, the results suggested that this motif was critical for the chito-oligosaccharide synthesis by the same protein. All the amino acid residues were located in the predicted central cytoplasmic loop domain that was suggested to be involved in the catalytic activity (18). These findings suggest that the motif of D,D,QXXRW may play an essential role for the transferase activity of GlcNAc to GlcA with β1→4 linkage in HA synthesis by HAS1 protein. Griffiths et al. (28) have shown that two aspartic acid residues were critical for the β1→4 addition of UDP-GlcA to oligosaccharide acceptors in K6C protein, which is one of the proteins that synthesize the K5 antigen of <i>Esherichia coli</i>, N-acetylmuramalosan, and suggested that these two residues were catalytically important for β-glycosyltransferase activity. One of these two aspartic acid residues corresponds to the residue Asp<sup>242</sup> which has been shown to be essential for both HA and chito-oligosaccharide synthesis in HAS1 protein. Thus, this finding also support our suggestion that the motif of D,D,QXXRW may be responsible for the β1→4 GlcNAc transferase activity.

Leu<sup>214</sup> on HAS1 protein was found to be essential for HA synthesis but not chito-oligosaccharide synthesis. It is located in the motif of SG,L on the predicted cytoplasmic central loop domains of all HAS proteins. All UDP-GTs that catalyze the addition of GlcA with β1→3 linkage have the motif similar to this but reverse in the direction within the UDP-GlcA binding domain. Thus, we predicted that the residue might be important for the transferase activity of GlcA to GlcNAc with β1→3 linkage in HA synthesis by HAS1 protein. If this is the case, the residue may not be required for chito-oligosaccharide synthesis. Since the residue Leu<sup>214</sup> is localized within the motif of D,D,QXXRW, catalytic sites for the transferase activities of GlcNAc and GlcA may be located closely each other on three dimensions and form the active pocket to function as both UDP-sugar binding sites and the catalytic sites.

Many attempts have been made to elucidate the HA biosynthetic mechanism. Previous studies have suggested that those for HA and other glycosaminoglycans are different and use different precursor pools of UDP-GlcNAc and UDP-GlcA (29, 30). The features of HA synthesis are as follows; HA can be synthesized in vivo in the absence of protein synthesis (31), and no covalently bound peptide could be found on the hyaluronan synthesized in vitro (32). Tunicamycin, an inhibitor of dolichol phosphate precursor synthesis, had no influence on HA synthesis (33, 34). The involvement of lipid intermediates in the synthesis has still been uncertain in eukaryotes, although it has been declined in Streptococci (35, 36). In prokaryote, the mechanism of HA synthesis was recently elucidated that streptococcal HAS enzymes conformed the complex with cardiolipin molecules and could synthesize HA in monomer (16). Here, we, for the first time, demonstrated that eukaryotic HAS protein has the capacity to synthesize HA without the addition of any other components except for the sugar nucleotide precursors and has both the glycosyltransferase activities of GlcNAc and GlcA, although it remains uncertain whether the proteins were active in monomer or formed the homopolymer. Prehm (37) proposed that HA synthesis required no primer molecule other than the substrates; chain initiation occurs with the substrate itself. This model seems consistent with the features of HA synthesis that we found in the present study. Although it was elucidated that HA could be synthesized by the hyaluronan synthases themselves in prokaryote and eukaryote, the mechanism of HA chain elongation still have remained unclear. One problem is the direction of HA chain elongation, and another one is whether a monosaccharide unit is alternatively added to HA chain or a disaccharide unit is conformed and linked to extend HA chain. Prehm (37) proposed that HA chain was elongated by the alternate additions of their reducing ends to the substrates with liberation of UDP. However, this model is not consistent with the recent reports (38) that HA synthesis occurs from the nonreducing end. The results in this study only suggested the least requirement for HA chain elongation, neither the initiation mechanism nor the direction of the chain synthesis. However, further development of this system may clarify some of these problems.

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