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Characterization of *Drosophila* Aspartic Proteases That Induce the Secretion of a Golgi-Resident Transferase, Heparan Sulfate 6-*O*-Sulfotransferase

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Alzheimer's β-secretase (BACE1), an aspartic protease, cleaves amyloid precursor protein to produce a neurotoxic peptide, amyloid-β, which plays a role in triggering Alzheimer's disease. We previously found that BACE1 also cleaves a glycosyltransferase, α2,6-sialyltransferase, as a physiological substrate. In the present study, we performed a BLAST homology search, identified two *Drosophila* aspartic proteases that are homologous to human BACE1, and isolated their cDNAs. The proteins encoded by the cDNAs were designated as DASP1 and DASP2, which exhibited 59% and 50% similarity to human BACE1, respectively. Each protein contained a pair of active site motifs (Asp/Thr or Ser/Gly), which is a common characteristic of aspartic proteases including BACE1. Although DASP1 and DASP2 did not contain an apparent transmenbrane domain, the proteases overexpressed in COS cells were localized in the Golgi area. Some of the DASP1 overexpressed in S2 cells was secreted, but none of the DASP2 was. DASP1 transcripts were expressed in the head of fruitflies, whereas DASP2 transcripts were mainly expressed in the body. When either DASP1 or DASP2 was coexpressed together with a Golgi-resident transferase, *Drosophila* heparan sulfate 6-*O*-sulfotransferase, the protease enhanced the secretion of the transferase from the cells, indicating that both DASP1 and DASP2 can induce the secretion of the 6-*O*-sulfotransferase.

Key words: aspartic protease, BACE1, glycosylation, glycosyltransferase, proteolytic cleavage.

Abbreviations: APP, amyloid precursor protein; APPL, amyloid precursor protein–like; BACE1, β-site amyloid precursor protein–cleaving enzyme 1; BDGP, Berkeley *Drosophila* Genome Project; DASP1, *Drosophila* aspartic protease 1; DASP2, *Drosophila* aspartic protease 2; dHS6ST, *Drosophila* heparan sulfate 6-*O*-sulfotransferase; DMEM, Dulbecco's modified minimal essential medium; FGF, fibroblast growth factor; HRP, horseradish peroxidase; HS6ST, heparan sulfate 6-*O*-sulfotransferase; PSGL-1, P-selectin glycoprotein ligand 1; RT-PCR, reverse transcription–polymerase chain reaction; ST6Gal I, α2,6-sialyltransferase I; TBS, Tris-buffered saline; Wnt, wingless.

The expression of glycans on complex carbohydrates is controlled mainly through the expression pattern of glycosyltransferases (*1–3*). Glycosyltransferases are localized in specific Golgi cisternae, or the endoplasmic reticulum, in an ordered way so that they can act sequentially on nascent glycan chains. The majority of glycosyltransferases are type II membrane proteins, and their structures are characterized by a luminal catalytic domain with a proteolysis-sensitive "stem region," followed by a transmembrane domain and a small cytoplasmic tail. The stem region is cleaved by endogenous protease(s) to generate a soluble form of the catalytic domain (*4–7*), which is secreted from the cells. Indeed, soluble forms of glycosyltransferases are detected in body fluids such as serum, urine, and milk. Some of the soluble forms in serum are used as diagnostic markers, the levels of which are correlated with the pathological status, such as inflammation or malignant transformation (*8*, *9*). In order to understand the molecular mechanism underlying the cleavage and secretion of glycosyltransferases, we have concentrated on identifying the endogenous protease(s) responsible for the cleavage, and we recently found that Alzheimer's β-secretase (β-site amyloid precursor protein-cleaving enzyme 1, BACE1) cleaves α2,6-sialyltransferase (ST6Gal I) (*10–12*).

BACE1 originally was identified as a pepsin-like membrane-bound aspartic protease that cleaved amyloid pre-

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cursor protein (APP) (*13–15*). BACE1 cleaves APP to produce a soluble NH_2-terminal fragment and a membrane-bound COOH-terminal fragment, which is further cleaved by γ-secretase, resulting in the production of the neurotoxic Aβ peptide. The Aβ peptide is then deposited in the brain, and the deposits are a hallmark of the pathology of Alzheimer's disease (*16*). Therefore β-secretase plays a crucial role in the initiation of Aβ formation and thus development of Alzheimer's disease. Northern blot analysis revealed that BACE1 mRNA is expressed in most organs including the brain (*13*, *15*), but physiological substrates for it other than APP had not been identified previously.

Quite recently, Lichtenthaler *et al.* reported that BACE1 also cleaves P-selectin glycoprotein ligand-1 (PSGL-1) (*17*). PSGL-1 is a core protein of *O*-glycans carrying the sialyl-LewisX determinant [Siaα2,3Galβ1,4(Fucα1,3) GlcNAc-], the ligand for P-selectin, which mediates leukocyte trafficking. Involvement of BACE1 in the cleavage of PSGL-1 and ST6Gal I suggests a possible regulatory role of the protease in glycoconjugate metabolism. Gain-of-function or loss-of-function experiments will likely be informative regarding this issue, and *Drosophila melanogaster* would be a useful model for such experiments. Although this organism does not appear to produce sialoglycoconjugates as major components, it well expresses many orthologues of mammalian transferases and glycan-related proteins (*18–21*). Indeed, several fruit fly mutations are attributed to abnormalities in these proteins, *e.g.*, UDP-glucose dehydrogenase in *Sugarless* (*22*), *N*-deacetylase/*N*-sulfotransferase in *Sulfateless* (*23*), and glypican, a core protein for heparan sulfate proteoglycans, in *Dally* (*24*, *25*). Biochemical analysis of these mutants has led to the notion that heparan sulfate glycosaminoglycans interact with morphogens and modify the signaling for embryogenesis. Analysis of *in vivo* cleavage of transferases by a BACE1 orthologue will be a promising way to address the biological relevance of the protease. In this context, we tried to identify aspartic proteases that are homologous to human BACE1 in *D. melanogaster* and found two candidates that enhanced the secretion of a sulfotransferase from cells.

MATERIALS AND METHODS

Drosophila BLAST Search—To identify *Drosophila* aspartic proteases that are homologous to human BACE1, we used the Fly BLAST released by the Berkeley *Drosophila* Genome Project (BDGP) (now one can access it at the Fly Base server: http://flybase.net/blast/). The full-length amino acid sequence of human BACE1 (GenBank accession no., AF204943) was used as a query sequence. We selected three candidate genes with high probability values (CG17134, CG13095, and CG6508; Table 1). The CG13095 cDNA clone was purchased from BDGP (clone ID, GH11417).

cDNA Cloning by Colony Hybridization—To isolate cDNA clones of CG17134 and CG6508, we screened a *Drosophila* 4–8 h embryo cDNA library, a generous gift from Nick Brown (University of Cambridge), by colony hybridization. The probes were PCR amplified from a *Drosophila* genomic library using 5′ primer ATGAGACAGTTGTTAGTTTT and 3′ primer TAAATAATCCGCCACCCTGG for CG17134 (1,172-bp fragment), and 5′ primer ATGGAAGTTGGCCAACTAAT and 3′ primer TCAAGTAAAGAATCTGGAGT for CG6508 (1,272-bp fragment). These DNA fragments were labeled with [^{32}P]-dCTP (ICN) using a DNA Labeling Kit (Takara Bio. Co., Tokyo, Japan). Each probe was hybridized with colonies (*ca.* 2×10^5) of the *Drosophila* embryo cDNA library as described by Sambrook *et al.* (*26*). Positive colonies were isolated, and their cDNA inserts were sequenced using a BigDye Thermal Cycle Sequencing Ready Reaction Kit and a PRISM 3100 DNA sequence analyzer (ABI). The inserts for CG17134 (DASP1) and CG13095 (DASP2) were PCR amplified using 5′ primer ATGAGACAGTTGTTAGTTTT and 3′ primer TTAATAATCCGCCACCCTGG for DASP1, and 5′ primer ATGTTCAAGACCATCGCTGT and 3′ primer 5′-TGCCAGTGCCATACTGGATG for DASP2. The PCR products were subsequently subcloned into the TOPO-TA cloning vector (Invitrogen).

Amino Acid Sequence Alignment—The cDNA and deduced amino acid sequences of DASP1 and DASP2 were analyzed using GENETYX-MAC software version 11.2.3 (Software Development Co., Ltd.). Hydropathy plots of the DASPs were prepared by the method of Hopp and Woods (*27*). The deduced amino acid sequences of human BACE1, DASP1, and DASP2 were aligned using the Clustal-W multiple sequence alignment system (http://clustalw.genome.jp/). A putative transmembrane domain was predicted using the Tmpred database (http://www.ch.embnet.org/software/TMPRED_form.html).

Reverse Transcription PCR—Total RNA was extracted from the head or body of fruitflies (Oregon-R strain) using Trizol reagent (Invitrogen) and then treated with DNase I at 37°C for 30 min. Approximately 900 ng of each total RNA was reverse transcribed in cDNA using a Superscript II RT-PCR Kit (Invitrogen). The target cDNA (150 ng) was PCR amplified using HotStar *Taq* DNA polymerase (Qiagen), and 5′ primer TTATGTGGCCAATGGTGAGG and 3′ primer TGCCAGTAGGCAGGAACGGA for DASP1, 5′ primer ATGTTCAAGACCATCGCTGT and 3′ primer TGCCAGTGCCATACTGGATG for DASP2, and 5′ primer ATGACCATCCGCCCAGCATACAGGCCCAAG and 3′ primer TGGCGGGTGCGCTTGTTCGATCCGTAACCG for ribosomal protein L32 (as a control).

Cell Cultures—Drosophila S2 cells were cultured at 25°C in Schneider's *Drosophila* medium (Invitrogen) supplemented with 210 μg/ml L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum. COS7 cells were cultured at 37°C in Dulbecco's modified minimal essential medium (DMEM) (Sigma) supplemented with 210 μg/ml L-glutamine, 110 μg/ml sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum in a 5% CO_2-humidified incubator.

Transient Expression of DASP1 and DASP2 in S2 Cells—The cDNA of DASP1 or DASP2 was inserted into the *Eco*RI site of the pMT-V5/His expression vector (Invitrogen) to yield pMT-V5/His-DASP1 or pMT-V5/His-DASP2, respectively. Each construct was transfected into *Drosophila* S2 cells using LipofectAmine 2000 (Invitrogen) according to the manufacturer's protocol. The transfected cells were incubated at 25°C for 6 h, and then $CuSO_4$ (final concentration, 500 μM) was added to the

medium to induce expression of the DASP protein. The cells were further incubated in the presence of Cu^{2+} for 18 h.

The amounts of DASP protein expressed in the cells and secreted into the culture media were determined by immunoblotting as follows. The cells were homogenized at 4°C in 1 ml of cell lysis buffer comprising 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, and a protease inhibitor cocktail (Sigma). The solubilized DASP protein was precipitated with 20 μl of a slurry of Ni-NTA (Qiagen) or heparin-agarose (Sigma). The soluble form of DASP in the culture medium was also precipitated with 20 μl of a slurry of each affinity resin. The proteins thus precipitated were subjected to SDS-PAGE on 4%–20% gradient gels (10 μl of a slurry of each resin/well) and then transferred to nitrocellulose membranes. The membranes were incubated with anti–V5-tagged IgG (1:1,000, Invitrogen) as a primary antibody, washed with TBS containing 0.1% Tween, and then incubated with HRP-conjugated goat anti-mouse IgG (1:1,000, Cappel). The antigen signal was detected using the Super-Signal West Dura Extended Duration system (Pierce).

Immunocytochemistry—The cDNA of DASP1 or DASP2 was inserted into the *Eco*RI site of the pcDNA3.1/myc-His expression vector (Invitrogen) to yield pcDNA3.1/myc-His-DASP1 or pcDNA3.1/myc-His-DASP2, respectively. COS7 cells were cultured on polylysine-coated microscopy cover slips. The cells were transfected with 1 μg of either expression construct together with 1 μg of pSVL-α2,6-sialyltransferase I (*7*), which contained the cDNA for α2,6-sialyltransferase, as a Golgi-marker protein. After 24 h culture, the cells were fixed in methanol at –20°C for 10 min. The fixed cells were pre-incubated with PBS containing 5% goat serum (blocking buffer) at room temperature for 45 min. The cells were then incubated with a mixture of mouse anti-myc monoclonal antibody (1:1,000, MBL) and rabbit anti–α2,6-sialyltransferase I polyclonal antibody (1:500, a generous gift from Dr. Karen J. Colley, University of Illinois, Chicago) in blocking buffer at room temperature for 1 h. After gentle washing with PBS, the cells were incubated with Alexa Fluor 546–conjugated goat anti–mouse IgG antibodies (1:100, Molecular Probes) and Alexa Fluor 488–conjugated goat anti–rabbit IgG (1:100, Molecular Probes) in blocking buffer at room temperature for 45 min. The stained cells were washed in PBS and then analyzed using an Axiovert 100 Minverted microscope LSM510 (Zeiss) equipped with a C-Apochromat (magnification, ×63; numerical aperture, 1.4) oil-immersion objective.

Pulse-Chase Analysis—Pulse-chase analysis was performed as described previously (*7*, *10*). The cDNA of *Drosophila* HS6ST (dHS6ST) tagged with the FLAG sequence (CTTGACTACAAGGACGACGATGACAAG, LDYKDDDDK) was inserted into the *Xho*I–*Bam*HI site of the pSVL expression vector (*7*) to yield pSVL-dHS6ST-FLAG. The construct was transfected into COS7 cells together with either pcDNA3.1/myc-His-DASP1 or pcDNA3.1/myc-His-DASP2. After 24 h culture, the cells were incubated in methionine- and cysteine-free DMEM (Invitrogen) at 37°C for 30 min. The cells were labeled in the same medium containing [^{35}S]methionine (100 μCi/ml, ICN) at 37°C for 1 h and then chased in 4 ml of DMEM containing 10% fetal bovine serum for 8 h. The cells were homogenized at 4°C in 1 ml of cell lysis buffer as described above. The dHS6ST in cell lysates and the soluble forms that were secreted into the supernatant were precipitated with 20 μl of a slurry of anti-FLAG M2 resin (Sigma). The precipitated dHS6ST was subjected to SDS-PAGE analysis on 4%–20% gradient gels (10 μl of a slurry of each resin/well). The radioactivity associated with dHS6ST was detected with a BAS-2000 image analyzer (Fuji Film).

RESULTS

BLAST Search for Drosophila Aspartic Proteases—Aspartic proteases including BACE1 contain two D(T/S)G sequences as active-site motifs (*28*). To identify aspartic proteases that contain these motifs, we performed a BLAST search using the complete amino acid sequence (501 residues) of human BACE1 as the query. As a result, we identified 11 candidate genes (Table 1). These 11 genes included a candidate (CG1548) that had already been annotated as *Drosophila* cathepsin D. We therefore attempted to isolate the cDNAs of the three genes (CG17134, CG13095, and CG6508) that exhibited higher probability values than the putative *Drosophila* cathepsin D.

cDNA Cloning—The hybridization probe for CG17134, the first candidate, was prepared by genomic PCR amplification and was used to screen a *Drosophila* embryo cDNA library. From 200,000 colonies screened, two positive clones were isolated. Sequence analysis revealed that one of the two clones contained the full-length cDNA of CG17134. The cDNA library similarly was screened for CG6508, the second candidate, but no positive clone was identified. In addition, we could not PCR amplify any DNA fragments of CG6508 using an embryonic cDNA template, suggesting that its cDNA was very rare or not expressed at the embryonic stage. An EST search on FlyBase for CG13095, the third candidate, revealed that a single clone (GH11417) contained the full-length cDNA of CG13095; we purchased this clone from BDGP. Therefore we obtained the full-length cDNAs of CG17134 and CG13095, which exhibited the two highest probability values.

Sequence Analysis of cDNAs—The deduced amino acid sequences of the proteins encoded by CG17134 and CG13095 are shown in Fig. 1A. CG17134 encoded a predicted protein of 391 amino acid residues with two potential *N*-glycosylation sites (Asn^{30} and Asn^{162}), whereas CG13095 ecoded a protein of 372 amino acid residues. The former protein was designated as DASP1 (*Drosophila* aspartic protease 1), and the latter as DASP2. The hydropathy plots for DASP1 (Fig. 1B) and DASP2 (Fig. 1C) indicated that each protein contained a putative signal sequence at the amino terminus. We aligned the amino acid sequences of the DASPs with that of human BACE1 (Fig. 1A) and found that (i) DASP1 is 25.8% identical and 58.6% similar with human BACE1, whereas DASP2 is 20.4% identical and 49.5% similar; (ii) each protein contains two D(T/S)G active-site motifs (amino acid residues 94–96 and 279–281 in DASP1 and 87–89 and 270–272 in DASP2); (iii) DASP1 has a putative heparin-binding sequence at amino acid residues 116–120; and (iv) unlike BACE1, neither DASP has a putative transmembrane domain.

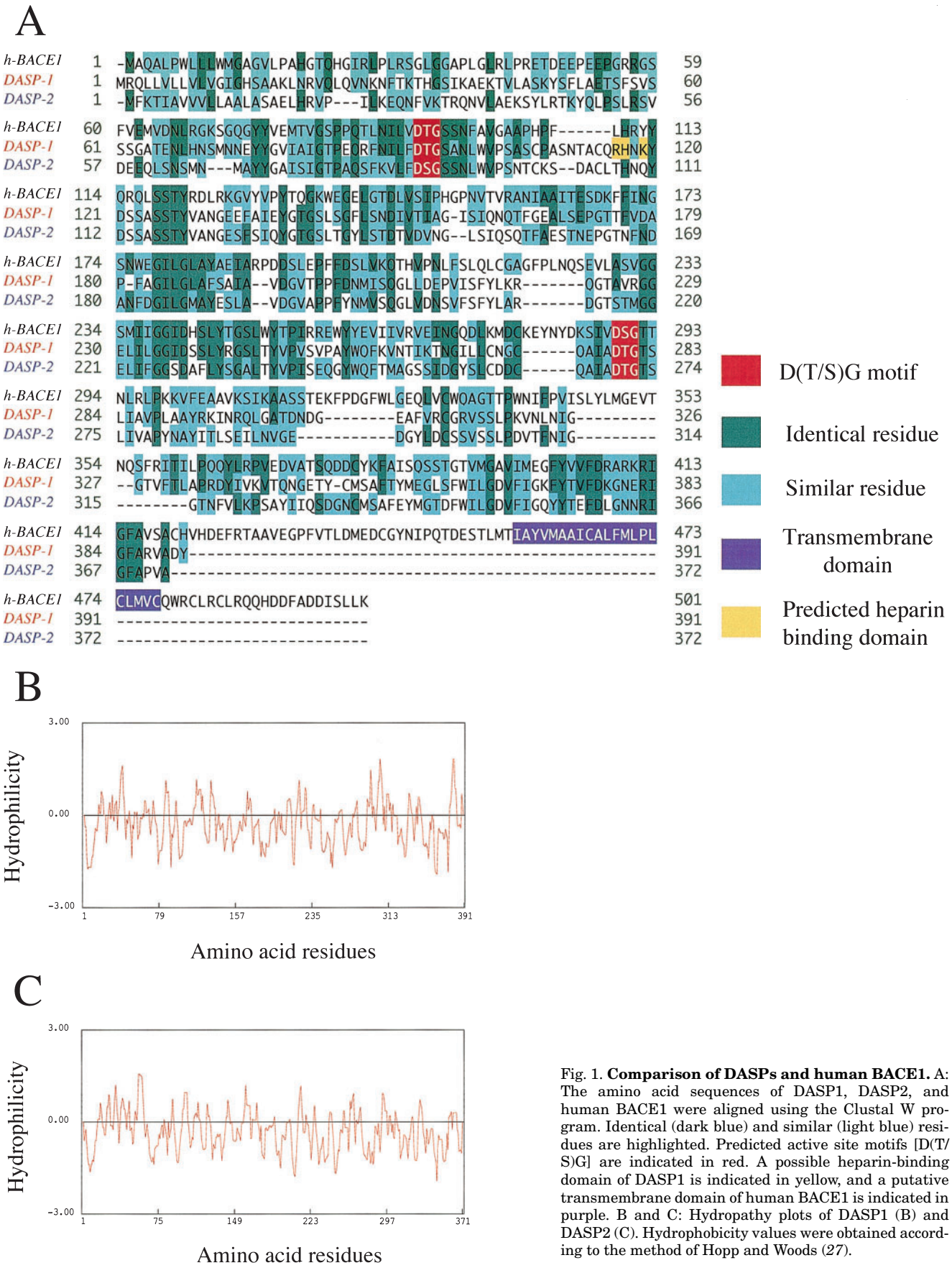

Fig. 1. **Comparison of DASPs and human BACE1.** A: The amino acid sequences of DASP1, DASP2, and human BACE1 were aligned using the Clustal W program. Identical (dark blue) and similar (light blue) residues are highlighted. Predicted active site motifs [D(T/S)G] are indicated in red. A possible heparin-binding domain of DASP1 is indicated in yellow, and a putative transmembrane domain of human BACE1 is indicated in purple. B and C: Hydropathy plots of DASP1 (B) and DASP2 (C). Hydrophobicity values were obtained according to the method of Hopp and Woods (*27*).

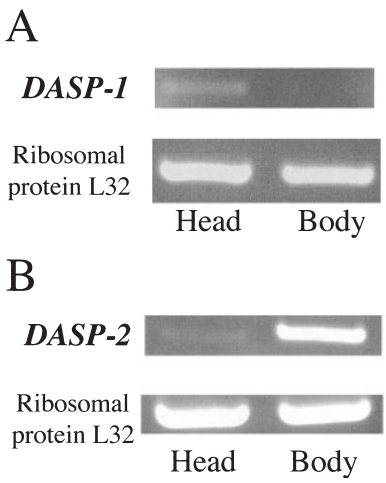

Fig. 2. **Expression of DASP mRNAs in *Drosophila* head and body.** Transcripts of DASP1 (A) and DASP2 (B) in *Drosophila* head and body were detected by RT-PCR. The transcript of ribosomal protein L32 is shown as a control.

mRNA Expression of DASP1 and DASP2 in Drosophila—We used RT-PCR analysis to investigate the expression of *DASP* mRNA. Total RNA was prepared from either the head or body of fruitflies. *DASP1* mRNA expression predominated in the head, with subtle expression in the body (Fig. 2A). In contrast, the *DASP2* transcript was predominant in the body, with slight expression in the head (Fig. 2B). These results indicate that *DASP1* and *DASP2* mRNAs exhibit distinct expression patterns in the head and body of *Drosophila*.

Subcellular Localization and Processing of DASP1 and DASP2 in Cultured Cells—Both DASPs had a putative signal sequence but lacked a transmembrane domain, suggesting that the DASP proteins were secreted from cells. To investigate this possibility, the DASPs were overexpressed in *Drosophila* S2 cells. The proteins were tagged with V5 and His sequences. DASPs of the expected molecular masses (ca. 50 kDa) were well overexpressed in the cells (Fig. 3A, cell lysate). The DASP1 secreted into the culture medium was precipitated with Ni-NTA and then detected by Western blot analysis using the anti-V5 antibody. An intense band of secreted DASP1 was observed in the 50-kDa region of an immunoblot (Fig. 3A, culture medium), indicating that DASP1 was actively secreted from S2 cells. The secreted DASP1 also was precipitated efficiently with heparin-agarose, suggesting that the protein's heparin-binding motif was functional. In contrast, we could not confirm any secretion of DASP2, although it was expressed as well as DASP1 was in the cells. DASP2 may be degraded in lysosomes. These results suggest that the intracellular

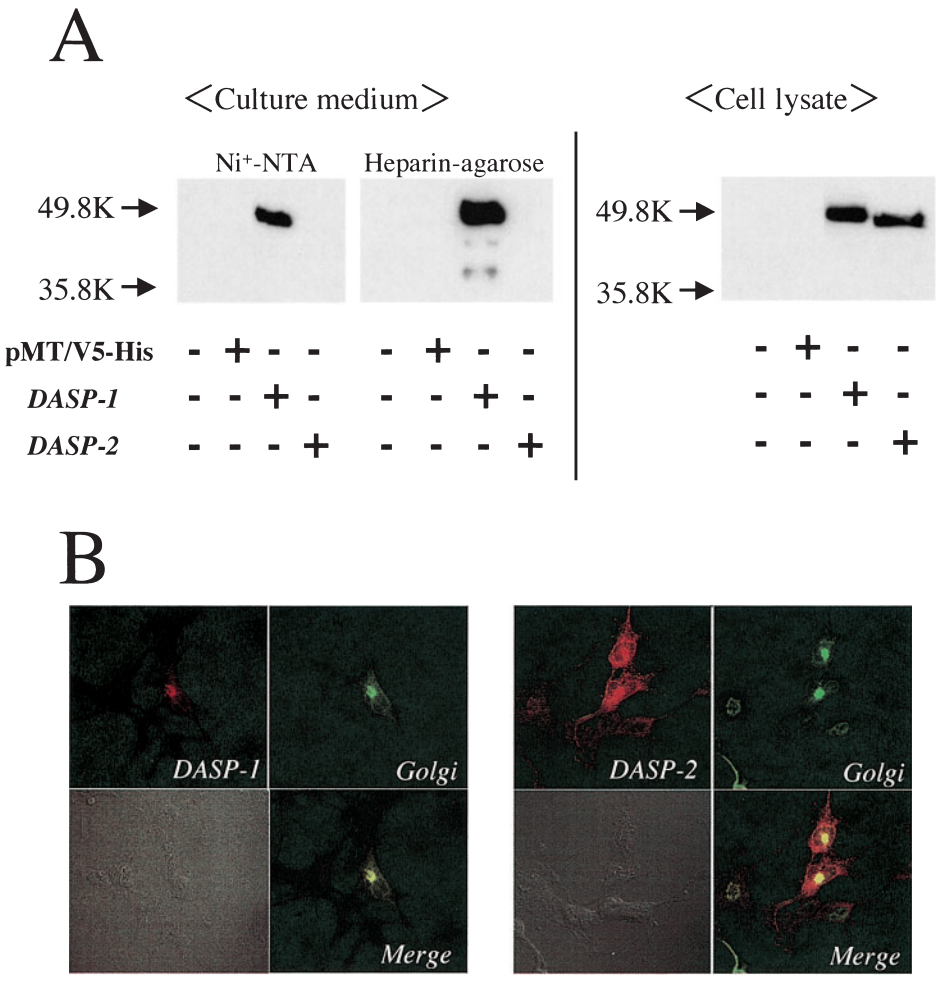

Fig. 3. **Overexpression of DASPs in cultured cells.** A: DASPs tagged with V5-His were overexpressed in *Drosophila* S2 cells. The overexpressed protein in the culture medium was precipitated with either Ni-NTA (left panel) or heparin-agarose (middle panel). The right panel indicates the expression levels of DASPs in cell lysates. DASPs were detected by Western blot analysis using anti-V5 antibody. B: DASPs tagged with myc-His were overexpressed in COS cells. Expression of DASP1 (left panel) and DASP2 (right panel) was detected by immunocytochemical analysis using anti-myc antibody (the red signal in the upper left quadrant of each panel). Cotransfected ST6Gal I was also detected as a Golgi marker (the green signal in the upper right quadrant of each panel). The two signals are merged in the lower right quadrants of the panels.

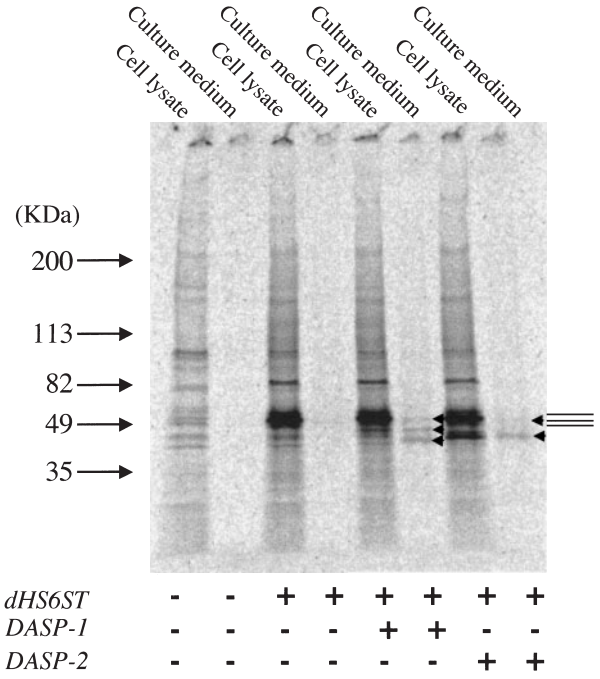

Fig. 4. **DASPs induced secretion of dHS6ST.** dHS6ST cDNA tagged with the FLAG sequence was cotransfected with either that of DASP1 or DASP2. The cells were labeled with [^{35}S] (100 μCi/ml) in methionine- and cysteine-free DMEM for 8 h. dHS6ST protein was immunoprecipitated from the cell lysates and culture medium using anti-FLAG antibody. Radiolabeled proteins were detected with a radioimage analyzer. The positions of major triplet bands for the cell lysate induced by the overexpression of dHS6ST are indicated by solid lines of the right of the panel. The bands generated for the supernatant upon cotransfection of dHS6ST with DASPs are indicated by arrowheads.

processing of DASP2 is quite different from that of DASP1.

To investigate the subcellular localization of each protease, myc- and His-tagged DASP1 or DASP2 was transiently expressed in COS cells. Confocal microscopy analysis revealed that DASP1 was localized in the Golgi area, where α2,6-sialyltransferase, a Golgi-marker protein, was also detected (Fig. 3B). DASP2 occurred not only in the Golgi area but also on the plasma membrane and reticular structures inside the cells (Fig. 3B). These results indicated that DASP1 and DASP2 exhibited distinct subcellular localizations and were differentially processed in the cells. In other words, DASP1 was localized mainly in the Golgi apparatus and was secreted from the cells, whereas DASP2 was distributed in various subcellular components and was not secreted.

Involvement of DASPs in Transferase Processing—We previously found that BACE1 cleaves both α2,6-sialytransferase and APP in the Golgi apparatus for the proteolytic processing of these substrates (*10*–*12*). Taking into account the structural similarity of DASPs with BACE1 as well as their localization in the Golgi, we speculated that DASPs cleave *Drosophila* Golgi proteins such as transferases and APPL, an orthologue of mammalian APP (*29*–*31*). Indeed, we found that a Golgi-resident transferase, *Drosophila* heparansulfate-6-*O*-sulfotransferase (dHS6ST), can be a substrate for DASPs. When

Table 1. **Eleven candidates drosophila orthologues of human BACE1.**

Gene	No. of a.a.	Probability value
CG17134 (*DASP-1*)	391	1.2e-24
CG13095 (*DASP-2*)	372	3.4e-24
CG6508	423	1.1e-22
CG1548 (*Cath D*)	392	1.2e-18
CG5863	395	2.0e-18
CG17283	465	7.4e-17
CG10872	405	4.2e-16
CG13374 (*pcl*)	407	4.5e-14
CG10104	404	1.5e-14
CG5860	370	3.0e-08
CG31661	393	6.7e-06

dHS6ST alone was overexpressed in COS cells, 50-kDa proteins appeared in the cell lysate (Fig. 4). The proteins seemed to be overexpressed dHS6ST (predicted molecular weight, 49,374). The apparent molecular size heterogeneity of the proteins was possibly due to posttranslational modifications including glycosylation. Cotransfection of either DASP1 or DASP2 with dHS6ST generated soluble proteins in the medium (Fig. 4). The molecular masses of the soluble proteins were smaller than those of dHS6ST overexpressed in the cell lysate, suggesting proteolytic cleavage of the transferase for secretion. We also noted that cotransfection of DASP1 with dHS6ST gave several bands corresponding to different molecular masses for the medium, which we did not observe upon cotransfection of DASP2 (Fig. 4). In view of these results, we conclude that DASP1 and DASP2 have functions homologous to that of BACE1 in terms of the processing or secretion of the transferase. In contrast, cotransfection of DASP1 or DASP2 did not enhance the secretion of APPL, another possible *Drosophila* substrate, or of human APP with the *Swedish* mutation (*32*, *33*), an authentic BACE1 substrate (data not shown). These results suggest that neither DASP1 or DASP2 is involved in APPL processing.

DISCUSSION

In the present study, we identified two candidates *Drosophila* aspartic proteases that are homologous to human BACE1, DASP1 and DASP2, and demonstrated that when these proteases were cotransfected with *Drosophila* HS6ST, they induced the secretion of the transferase from COS cells.

HS6ST catalyzes the 6-*O*-sulfation of glucosamine residues in heparan sulfate proteoglycans (*34*, *35*). Sulfation of the proteoglycans is a crucial modification for their interaction with various growth factors and morphogens (*36*, *37*). Indeed, their signals are modified by sulfotransferases and sulfatases, which control the sulfation of the proteoglycans. Wang *et al.* recently reported that avian 6-*O*-sulfatase (Qsulf1) regulates FGF and Wnt signaling through hydrolysis of the 6-*O*-sulfate residues on heparan sulfate proteoglycans (*38*). Kamimura *et al.* reported that a reduction in HS6ST expression due to RNA interference disrupts tracheal branching in *Drosophila* because of the impairment of FGF signaling (*34*). In the present study, we demonstrated that DASP1 and DASP2 induce the secretion of dHS6ST, suggesting that

DASPs have a regulatory role in the expression of the transferase. The soluble forms of dHS6ST secreted from COS cells exhibited lower molecular weights than the membrane-bound forms, suggesting proteolytic cleavage of the transferase for secretion. DASPs may cleave dHS6ST directly, but currently we cannot exclude other possibilities, *e.g.*, DASPs may activate some other protease(s) for the cleavage of dHS6ST. Purification and characterization of DASPs will be required to clarify how DASPs are involved in the secretion mechanism. Another important question to be addressed is whether or not the DASP-dependent cleavage of dHS6ST affects 6-*O*-sulfation. In the case of α2,6-sialyltransferase I (ST6Gal I), overexpression of BACE1 in cultured cells enhanced ST6Gal I secretion and altered the level of α2,6-sialyl residues, the products of the sialyltransferase (*10–12*). DASPs also may regulate the 6-*O*-sulfation of heparan sulfates in *Drosophila* and may affect 6-*O*-sulfation-dependent signals. Gain-of-function or loss-of-function experiments involving *Drosophila* as a model will shed light on the biological functions of DASPs *in vivo*.

DASPs do not have a putative transmembrane domain, but these proteases still appear to cleave dHS6ST. This was consistent with our recent observation that soluble BACE1, which lacked the transmembrane and cytosolic domain, could cleave the membrane-bound form of ST6Gal I, when these molecules were overexpressed in COS cells (manuscript in preparation, Hattori *et al.*). Some glycosyltransferases are likely to be susceptible to soluble forms of BACE1 and DASPs, although the exact molecular mechanism underlying the susceptibility is currently unknown. Immunocytochemical analysis showed that DASP1 was localized mainly in the Golgi area and was secreted from cells, whereas DASP2 had additional localization sites (including the plasma membrane and reticular structures in the cytoplasm) and was not secreted. DASPs showing different subcellular localizations may have distinct substrates, which are relevant to their specific functions. A further search for possible substrates for DASPs will be important for understanding their biological relevance. DASP1 and DASP2 likely contain unique sorting signals in their sequences. Scholefield *et al.* reported that BACE1 binds to heparan sulfate proteoglycans, and that the binding affects the activity of BACE1 (*39*). A heparin-binding sequence of DASP1 may be responsible for its localization, *e.g.*, the sequence may bind heparan sulfate proteoglycans in the Golgi apparatus and/or the binding complex may be secreted from the cells. A chimeric protein, in which the heparin-binding sequence is introduced into DASP2, could provide a clue regarding the mechanism underlying the difference in sorting.

Syntichaki *et al.* reported that *Caenorhabditis elegans* aspartic proteases Asp-3 and Asp-4, which may be nematode orthologues of DASPs, are required for neurodegeneration in *C. elegans* (*40*). Although the involvement of DASPs in neurodegeneration remains to be elucidated, we examined their possible roles in the cleavage of APPL or human APP with the Swedish mutation. However, no cleavage of the substrates was detected under the overexpression conditions with which dHS6ST was cleaved. The pathophysiological function of DASPs may be distinct from that of human BACE1.

In conclusion, we have identified two functional *Drosophila* aspartic proteases, DASP1 and DASP2, which induce the cleavage of dHS6ST and its secretion from cells as a possible control mechanism of glycan expression.

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