(*11*). Typical chromatograms showed seven major peaks (Fig. 3). On the basis of a limited number of bioassays ($n = 3$), we have tentatively assigned DH activity to two different peaks, whereas the ADH activity is associated exclusively with one peak ($n = 8$). The purified ADH is highly potent, with 1.0 CC equivalent inhibiting secretion by 70 ± 8% (mean ± SE). With the standard gradient (*11*), the ADH peak elutes 6.4 min before the AKH peak (*12*). To obtain sufficient material to bioassay required that pooled samples of ten or more CC be chromatographed. In general, however, the use of pooled samples masks individual variability to the extent that differences between control and experimental treatments are often concealed (*9*). This difficulty was addressed by modifying the HPLC method to optimize ADH separation and by increasing the sensitivity to the point where the CC of individual crickets could be successfully chromatographed (*13*). Crickets were first treated as described for the preparation of hemolymph extracts with the exception that the desiccation time was increased to 48 hours to maximize the effect. The use of individual CC precluded accurate bioassays; therefore a sample of the ADH peak recovered by the standard HPLC method was reinjected into the column to serve as a marker (Fig. 4A). Individual CC from control (Fig. 4B) and dehydrated (Fig. 4C) crickets were then chromatographed over the same gradient. The size of the ADH peak in the CC of dehydrated crickets was significantly smaller than in controls, whereas, overall, the other peaks were not changed (Fig. 4, B and C). Integration of each ADH peak showed that the area (*14*) decreased from 68.7 ± 9.4 (control, $n = 6$, mean ± SE) to 39.5 ± 5.3 (dehydrated, $n = 6$), a 42% decrease [$t(10) = 2.693$, one-tailed; $P < 0.05$].

We have developed an in vitro preparation of the perfused rectum of *Acheta* that is identical in all important respects to that used for the Malpighian tubules (*15*). Using this preparation as a bioassay, we showed that crude CC homogenate increased fluid absorption by the rectum sixfold, that is, it acted as an ADH (*16*). The HPLC-purified ADH, which inhibits Malpighian tubule secretion, has no effect on reabsorption in this preparation ($n = 8$) and therefore is not the same as the CC factor that increases fluid absorption by the rectum (*16*).

These experiments show the presence in insects of an ADH that directly inhibits Malpighian tubule secretion in vitro without altering rectal reabsorption. In the cricket *Acheta domesticus*, ADH is released from specific neurosecretory axon endings in the CC in response to water stress and is transported from the CC to the tubules by way of the hemolymph. The existing model for the control of Malpighian tubule function (*2*) may not be universal among phytophagous insects, since we have demonstrated that in addition to the controls described by the model, *Acheta* possesses a neurohormone capable of directly inhibiting tubule secretion.

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12. The AKH peak was identified by the hyperlipemic response this fraction produced in *Locusta migratoria*, as described in (*11*).
13. The extracts and column were identical to those described in (*11*). Solvent A (water) and solvent B (60% acetonitrile in water) were optically balanced against each other with TFA. Approximate concentrations of TFA were 0.427% (solvent A) and 0.350% (solvent B). The solvents were applied as a linear gradient (25 to 55% B in 20 min; 1.0 ml/min of flow rate) and the eluant monitored at 210 nm.
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1 April 1988; accepted 23 June 1988

Coding of Two Sphingolipid Activator Proteins (SAP-1 and SAP-2) by Same Genetic Locus

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Several complementary DNAs (cDNAs) coding for sphingolipid activator protein–2 (SAP-2) were isolated from a λgt-11 human hepatoma library by means of polyclonal antibodies. The nucleotide sequence of the largest cDNA was colinear with the derived amino acid sequence of SAP-2 and with the nucleotide sequence of the cDNA coding for the 70-kilodalton precursor of SAP-1 (SAP precursor cDNA). The coding sequence for mature SAP-2 was located 3′ to that coding for SAP-1 in the SAP precursor cDNA. Both SAP-1 and SAP-2 appeared to be derived by proteolytic processing from a common precursor that is coded by a genetic locus on human chromosome 10. Two other domains similar to SAP-1 and SAP-2 were also identified in SAP precursor protein. Each of the four domains was approximately 80 amino acid residues long, had nearly identical placement of cysteine residues, potential glycosylation sites, and proline residues. Each domain also contained internal amino acid sequences capable of forming amphipathic helices separated by helix breakers to give a cylindrical hydrophobic domain that is probably stabilized by disulfide bridges. Protein immunoblotting experiments indicated that SAP precursor protein (70 kilodaltons) as well as immunoreactive SAP-like proteins of intermediate sizes (65, 50, and 31 kilodaltons) are present in most human tissues.

SPHINGOLIPID ACTIVATOR PROTEINS (SAPs) are small (8 to 13 kD), heat-stable proteins required for the hydrolysis of sphingolipids by specific lysosomal hydrolases (*1*). To date, three different SAPs have been identified. SAP-1 activates the hydrolysis of cerebroside sulfate, GM1 ganglioside, and globotriaosylceramide by arylsulfatase A (E.C. 3.1.6.1), acid β-galactosidase (E.C. 3.2.1.23), and α-galactosidase (E.C. 3.2.1.22), respectively (*2*). Human deficiency of SAP-1 results in tissue

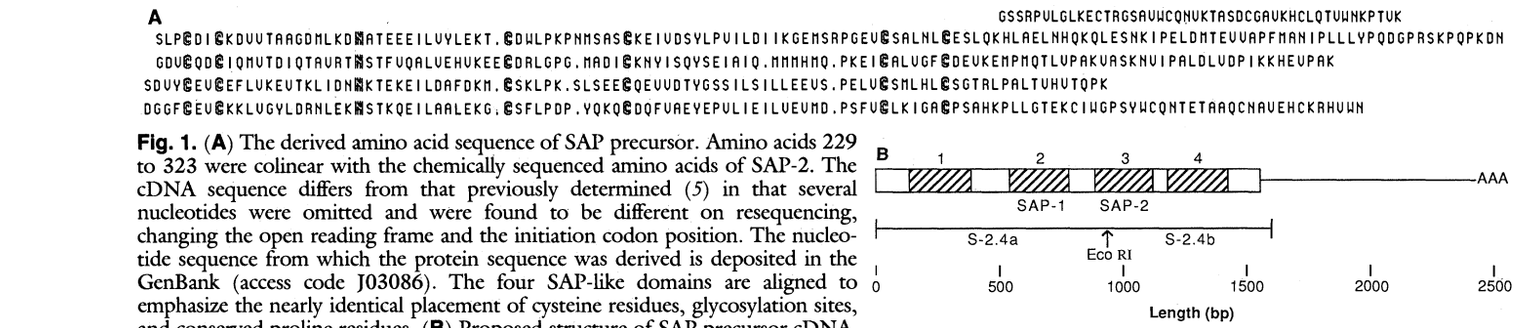

Fig. 1. (**A**) The derived amino acid sequence of SAP precursor. Amino acids 229 to 323 were colinear with the chemically sequenced amino acids of SAP-2. The cDNA sequence differs from that previously determined (*5*) in that several nucleotides were omitted and were found to be different on resequencing, changing the open reading frame and the initiation codon position. The nucleotide sequence from which the protein sequence was derived is deposited in the GenBank (access code J03086). The four SAP-like domains are aligned to emphasize the nearly identical placement of cysteine residues, glycosylation sites, and conserved proline residues. (**B**) Proposed structure of SAP precursor cDNA. The open reading frame of the precursor is shown as an open box with the regions coding for mature SAP-1 and SAP-2 indicated. The cross-hatched regions of the open reading frame designate the four SAP-like domains discussed in the text. Domains 1 to 4 correspond to amino acid residues 47 to 130, 182 to 262, 298 to 377, and 392 to 474, respectively. The internal Eco RI site that was used to generate fragments S-2.4a and S-2.4b is also shown. The size of 2.5 kb shown for the cDNA includes approximately 900 bp of 3′ untranslated sequence based on the size of a partially sequenced clone containing a polyadenylation signal and a poly(A) tail (*17*). This size estimate corresponds well to that reported by Collard *et al.* (*22*) for SGP-1 cDNA.

accumulation of cerebroside sulfate (and possibly other glycolipids) and results in a clinical picture resembling metachromatic leucodystrophy (*3*). A cDNA coding for SAP-1 was cloned and sequenced (*4*); recently a longer cDNA coding for the 70-kD precursor of SAP-1 was sequenced (*5*).

A second activator protein, SAP-2, activates the hydrolysis of glucosylceramide, galactosylceramide, and sphingomyelin by β-glucosylceramidase (E.C. 3.2.1.45), galactosylceramide β-galactosidase (E.C. 3.2.1.46), and sphingomyelinase (E.C. 3.1.4.12), respectively (*6–8*). SAP-2 appears to differ from SAP-1 in mechanism of activation because it interacts with the enzyme β-glucosylceramidase, raising the maximum velocity and lowering the Michaelis constant (*9, 10*), whereas SAP-1 appears to interact only with sphingolipid substrates by solubilizing them for hydrolysis (*11*). Deficiency of activator A_{1a} (which is identical to SAP-2) has been reported in a single patient with a variant form of Gaucher's disease (*12*).

A third activator protein, SAP-3, is a specific activator for the hydrolysis of ganglioside GM2 by β-*N*-acetylgalactosaminidase A (E.C. 3.2.1.52) and is deficient in a variant form of GM2 gangliosidosis (AB variant) (*13*).

Studies of the proteolytic processing of SAP-1 and SAP-2 have shown that both proteins are produced from large (65 to 73 kD) precursors that are extensively glycosylated (*14*). Proteolytic processing generates the activator proteins that appear to be localized primarily within lysosomes (*15*). We report here the cloning of a cDNA for SAP-2 and show that a common protein precursor gives rise to both SAP-1 and SAP-2. Our evidence suggests that a single genetic locus codes for SAP-1, SAP-2, and perhaps two other SAP-like proteins of similar size and structure. We also show that the SAP precursor and a family of SAP proteins of larger size occur in various human tissues.

A human hepatoma cDNA expression library in λgt-11 was used for the isolation of SAP-2 clones that were screened with antibody to SAP-2 as described (*16*) to select positive plaques. After purification to homogeneity, the insert from one phage was isolated and used to rescreen the library by plaque hybridization; the largest cDNA (S-2.4, 1.6 kb) was selected and sequenced.

Because the hepatoma tissue used in the library construction had been grown in athymic mice, dot blot experiments were performed to establish the human origin of these clones. Labeled probe S-2.4b hybridized strongly to human DNA and only faintly to mouse DNA, indicating its human origin (*17*).

Clone S-2.4 was chosen for sequence analysis because of its large size and the presence of an internal Eco RI site that cleaved the insert into nearly equal pieces. After subcloning the fragment into the vector M13, it was sequenced by the dideoxy chain-termination method. The nucleotide sequence of S-2.4 was colinear with 25 amino acids determined by chemical sequencing of the amino terminus of mature SAP-2.

The deduced 80–amino acid sequence of SAP-2 is identical to that reported by Kleinschmidt *et al.* (*18*) who chemically sequenced an activator protein A_{1a} that was isolated from the spleen of a patient with Gaucher's disease (*19*). The sequence of S-2.4 was also colinear with that previously determined for the SAP-1 precursor cDNA (*5*). The stretch of sequence coding for mature SAP-2 is located 3′ to the coding sequence for mature SAP-1 (Fig. 1). The carboxyl terminus of SAP-1 has not been precisely identified, but SAP-1 is estimated to be 80 amino acid residues long (*5*). In the SAP precursor cDNA, the SAP-1 and SAP-2 coding sequences are separated by approximately 108 nucleotide residues, are in the same open reading frame, and are not separated by a stop codon. This fact indicates that SAP-1 and SAP-2 are generated by proteolytic processing of the same large molecular weight precursor (SAP precursor).

Protein immunoblotting was performed on a variety of human tissue samples with the use of monospecific antibodies to SAP-1 or SAP-2 (*17*) to determine the presence and distribution of SAP precursor. A series of immunoreactive proteins migrating as doublets (Fig. 2) was detected (70, 65, 50, and 31 kD) by both antisera. However, no cross-reactivity was found when antibodies to SAP-1 were blotted against pure mature SAP-2 (12 kD) or vice versa, indicating that the proteins larger than 30 kD contain antigenic determinants for both SAP-1 and SAP-2. The highest concentrations of the larger SAP proteins in human tissues were in brain and testis, but all organs examined (kidney, spleen, and liver) contained a set of similar immunoreactive bands (*17*).

The human locus encoding SAP-1 and SAP-2 has been mapped by somatic cell hybridization to chromosome 10 (*20*). In situ hybridization with a SAP-1 probe showed that the SAP-1 locus was localized to a single site on the long arm of chromosome 10 (q21–q22) (*21*).

Recently, Collard *et al.* (*22*) reported the cloning and nucleotide sequence of a sulfated glycoprotein (SGP-1) secreted by rat Sertoli cells; SGP-1 is similar in protein sequence (76%) to the human SAP precur-

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sor. In rat testis, SGP-1 is a major secretory glycoprotein that is posttranslationally modified to a 70-kD form before secretion into the extracellular space (*23*). It has been suggested that SGP-1 could function as a glycolipid transfer protein in spermatogenesis (*22*). Unlike human SAP precursor, SGP-1 is apparently not processed to smaller sized products but is secreted into the adluminal compartment of the seminiferous tubules. SGP-1 contains a proline-rich sequence (31 amino acids), located between SAP-like regions three and four (*22*), that is not present in human SAP precursor and may contribute to the different trafficking patterns, since proline-rich proteins are chiefly secretory proteins (*24*). Other differences between the cDNAs coding for rat SGP-1 and human SAP precursor include 66 additional 5′-most nucleotides in the SGP-1 cDNA coding for a 16-residue signal peptide, an initiation codon, and 27 5′ untranslated nucleotide residues.

Collard *et al.* (*22*) have pointed to the presence of four domains in SGP-1 protein that bear a close resemblance to SAP-1. Kleinschmidt *et al.* (*18*) also noted the similarity in amino acid sequence between SAP-1 and SAP-2. In the human SAP precursor, our analysis also reveals four structurally similar domains (Fig. 1), the internal two of which are SAP-1 and SAP-2. In addition to the nearly identical placement of cysteine residues (six in each domain), potential glycosylation sites, and proline residues at proposed turns at the ends of helical regions (Fig. 3), the four domains are also remarkably similar when their possible internal structures are compared. Each of the four domains has an approximate overall length of 80 amino acid residues; lysine residues occur at, or near, seven of the eight boundaries and may be proteolysis sites. At the eighth boundary region an arginyl-leucyl dipeptide is present. Helical wheel depictions (Fig. 3) of each domain indicate that at least three internal sequences (at approximately amino acid residues 1 to 18, 22 to 39, and 43 to 60 in each domain) are capable of forming amphipathic helices separated by helix breakers (glycosylation at position 21 and proline residues) with cysteine residues properly positioned to form internal disulfide bridges in the hydrophobic interior. A rigid disulfide bridge–stabilized structure might be expected because SAP-1, SAP-2, and SAP precursor are all stable when boiled (*17*).

Wynn (*25*) has proposed a triple–binding domain model for the interaction of SAP-1 with GM1-ganglioside, sulfatide, and globotriaosylceramide. His model suggests two, or possibly three, helical regions that could associate to form a cylindrical hydrophobic domain. One could be the interaction between the oligosaccharide of the protein and the plane of the hydroxyl groups of the lipid; another could be between a lysine (or other positive charge) and the negative charge on the lipid. Our studies on the prediction of secondary and tertiary structures of SAP-2 and other SAP-like domains indicate a hydrophobic pocket for each, similar to SAP-1. Sheh *et al.* (*26*) have proposed a model for SAP-2 (heat-stable factor) on the basis of data from nuclear magnetic resonance (NMR) spectroscopy that indicate a conformationally rigid hydrophobic pocket and a compact tertiary struc-

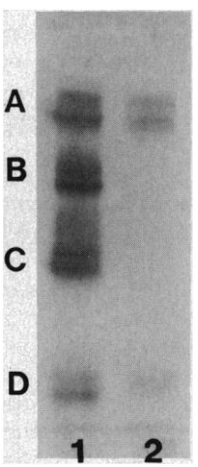

Fig. 2. Protein immunoblot analysis of duplicate tissue preparations with antibody specific for SAP-1. Human spleen was boiled in water for 10 min, centrifuged (8000*g*, 10 min), and portions of supernatant taken for SDS–polyacrylamide gel electrophoresis (12.5% acrylamide concentration) (lane 1). A partially purified sample of SAP precursor was prepared by collection of fractions of the boiled spleen extract chromatographed on a Sepharose 6B column and then analyzed by electrophoresis (lane 2). High molecular weight standards (not shown) were also run to define the approximate molecular weights of forms A, B, C, and D (70, 65, 50, and 31 kD, respectively). A similar pattern appeared when antisera to SAP-2 were used (*17*). Monospecific rabbit antisera to SAP-1 or SAP-2 were prepared essentially as described (*5*). SAP-1 was isolated from the liver of a patient with GM1 gangliosidosis by a series of column chromatographic steps and used for immunization; monospecific antibodies were purified from serum by passage through a SAP-1 affinity column prepared by linking SAP-1 to a cyanogen bromide–activated Sepharose 4B column and assessing monospecificity by protein immunoblotting. A similar procedure was used to obtain monospecific antibody to SAP-2, isolating pure SAP-2 from the spleen of a patient with Gaucher's disease (*29*). The blotting and staining method used is described in (*16*). SAP-1 and SAP-2 migrated at the front on this gel and are not shown.

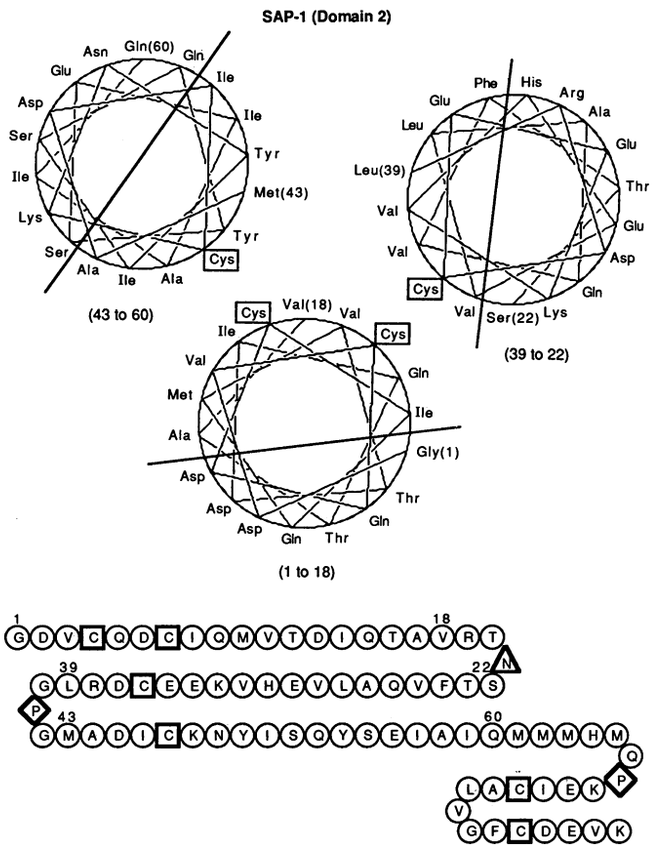

Fig. 3. Helical wheel and two-dimensional projections for SAP-1 (domain 2). (**Top**) Three wheels depicting three potential amphipathic helices of SAP-1 beginning with its amino-terminal residues 1 to 18, 39 to 22 (residues 22 to 39 inverted), and residues 43 to 60. Cysteine residues are boxed and the hydrophobic interior faces are indicated by lines through each wheel. (**Bottom**) A two-dimensional model of SAP-1 indicating the six cysteines (boxes), the single potential glycosylation site (triangle), and the conserved proline residues (diamonds). Similar analyses of the other three domains reveal nearly identical structures for each with regard to potential placement and length of amphipathic helices and position of glycosylation sites, cysteine residues, and helix breakers.

ture, which is in keeping with the structure proposed here. Analyses are in progress to determine whether unique amino acid sequences underlie the different activities of SAP-1 and SAP-2.

In analogy with other proteins with replicated domains (*27*), the four domains in SAP precursor apparently arose through duplication of an ancestral gene segment. Preservation of a similar tertiary structure of the four modular SAP units in SAP precursor during evolution is evidence for an important functional role for these units. The discovery of two different sphingolipid storage diseases in man that are genetically inherited and are caused by deficiency of SAP-1 and SAP-2 (*3, 11*) underscores this point. It appears likely that the SAP-like polypeptides that could arise from domains one and four from proteolysis of SAP precursor may also be functionally important because they are structurally similar to SAP-1 and SAP-2 and are bounded by lysyldipeptide linkages (*28*).

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30. We thank A. Ertl and M. Donoviel for technical assistance and M. Collard and M. Griswold for supplying a copy of their manuscript before publication. Supported in part by NIH grants NS 08682 [Javits Neuroscience Investigator Award (J.S.O.)], HD 18983 (J.S.O.), DK 38795 (D.A.W.), NS 11665 (A.L.F.), HO 4612 (A.L.F.); training grant PHS DK 07318 (K.A.K.); and March of Dimes Research Foundation Clinical Research grant No. 6-413 (D.A.W.).

28 April 1988; accepted 5 July 1988

"According to the voice-stress analyzer, he's <u>not</u> going to lower taxes."