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Genetics

Evidence for two dissimilar polypeptide chains in the β_2 subunit of hexosaminidase

(β-*N*-acetylglucosaminidase/GM_2 gangliosidosis/Tay–Sachs disease/Sandhoff disease)

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ABSTRACT **The major isoenzymes of human hexosaminidase have the structures $\alpha\beta_2$ (hex A) and $2\beta_2$ (hex B). In this study, we present evidence that the β_2 subunit of hex B and hex B_A (the form of hex B derived from hex A) is composed of two nonidentical polypeptide chains. We have called these chains β_a and β_b. They have similar molecular weights (25,000) but have pI values that differ by 1 unit. We have used a two-dimensional analytical gel electrophoresis method in combination with peptide mapping to compare the primary sequence structure of the two β chains. In this method, the polypeptide chains of hex B or hex B_A were first separated by isoelectric focusing in 8.5 M urea. The separated chains were subjected to partial proleolytic digestion in the stacking gel of a second NaDodSO$_4$/polyacrylamide gel with subsequent separation of peptides by electrophoresis into the second gel. Partial digestion by protease V8 or papain showed that the β_a and β_b species have distinct primary structures, neither of which was similar to that of the α chain. On the basis of these results, we suggest that the β_2 subunit of hexosaminidase has the structure of $\beta_a\beta_b$. The possibility that the distinct β chains are encoded by a single gene is discussed in the light of genetic and other data.**

Hexosaminidase (β-*N*-acetylglucosaminidase, EC 3.2.1.30) is a multimeric lysosomal enzyme. In humans, the enzyme is composed of two nonidentical polypeptide chains, α and β (1–3), that have apparent molecular weights of 50,000 and 25,000, respectively (4, 5). These chains are encoded by unlinked structural genes, with the α chain having been mapped to chromosome 15 and the β chain having been mapped to chromosome 5 (6, 7). Both chains associate to form several isoenzymes, of which hexosaminidase A (hex A) and hexosaminidase B (hex B) are the major tissue forms. In previous studies, we have shown that hex A from human placenta has the structure $\alpha\beta_2$ (4). In contrast, hex B and hex B_A, formed by the merthiolate-induced dissocation of hex A, are composed of two β_2 subunits ($2\beta_2$) that are linked by a least one disulfide bridge (4, 8).

Inherited disorders have been described in which there is a deficiency of hexosaminidase activity (9). In one of these, Tay–Sachs disease, there is a specific deficiency of the α chain, while Sandhoff disease is associated with a deficiency of the β chain (5). These disorders may have resulted from mutations of the corresponding structural genes (10, 11).

In this study, we provide biochemical evidence that β_2 subunits are comprised of two distinct polypeptide chains that have similar molecular weights of 25,000 but differ in pI and in peptide patterns after partial proteolytic digestion. The significance of this finding is discussed with regard to the hex A and hex B subunit structure and to the anomaly posed by the conflicting genetic data.

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MATERIALS AND METHODS

Materials. Acrylamide, NaDodSO$_4$, and other chemicals for polyacrylamide gels were obtained from Bio-Rad. Sigma supplied dithiothreitol, Nonidet P-40, papain type IV, guanidine·HCl (grade 1), and sodium iodoacetate. Aristar urea was obtained from British Drug House (Poole, England). Ampholines and Multiphor isoelectric focusing apparatus were purchased from LKB. Eastman supplied 2-mercaptoethanol. Protease V8 from *Staphylococcus aureus* was obtained from Miles. Other chemicals were of reagent grade.

Preparation of Hexosaminidase. Hex A and hex B were purified from pooled normal human placentas (4) and stored in 0.01 M phosphate buffer. Hex B_A was prepared from hex A by treatment with merthiolate and chromatography on DEAE-Sepharose (4). Each isozyme was determined to be electrophoretically pure by native and NaDodSO$_4$/polyacrylamide gel electrophoresis (4, 8).

Electrophoresis Techniques. Samples were prepared for electrophoresis by precipitating 300 μg of purified enzyme with 4 vol of acetone. The precipitate was washed twice with 80% acetone in distilled deionized water. It was dried and dissolved in 100 μl of 7 M guanidine·HCl/0.5 M Tris·HCl/2 mM EDTA adjusted to pH 8.5 with glacial acetic acid/50 mM dithiothreitol (added as a solid just before use). The solution was incubated for 2 min at 100°C and for 3 hr at 37°C. Solid Na iodoacetate was added to a final concentration of 150 mM. The solution was incubated for an additional hour at room temperature in the dark. After the incubation, 10 μl of 2-mercaptoethanol was added, and the enzyme was precipitated by adding 1 ml of absolute ethanol/1% glacial acetic acid at −20°C. The precipitate was washed three times with 70% ethanol and dried. Samples to be focused were dissolved in 50 μl of 10 M urea (deionized)/3% Nonidet P-40 just prior to application (longer exposure to urea caused an increase in microheterogeneity). Half of the dissolved precipitate, 25 μl, was applied to small O rings placed on the gel.

The polypeptide chains of hexosaminidase were separated by NaDodSO$_4$/polyacrylamide gel electrophoresis (12) or by isoelectric focusing on 12 × 25 cm × 2 mm polyacrylamide gel slabs (13). Two-dimensional separations using isoelectric focusing (1.2 mm thick) in the first dimension and NaDodSO$_4$/polyacrylamide gel electrophoresis (1.6 mm thick) in the second were carried out by a method modified from ref. 14. The focusing slabs contained 8.5 M deionized urea, 2.5% Nonidet P-40, and a mixed ampholine solution of pH 3.5–10, 2.5–4, 4–6, 6–8, 3:1:1:1 (vol/vol). The pH curves for the focusing gels were determined by cutting a 1-cm strip from one end of the gel into 1-cm squares and crushing them in small test tubes containing

Abbreviations: hex A and hex B, hexosaminidase A and B, respectively.

1 ml of distilled deionized water. The pH for each tube was determined after two hours incubation.

Peptide Mapping. Peptide mapping was accomplished by a combination of partial proteolytic digestion and an extension of the two-dimensional polyacrylamide gel procedure described by Bordiev and Crettol-Jarvinen (15). In this method, a protein mixture is first resolved into individual species by $NaDodSO_4$/polyacrylamide gel electrophoresis. The separated polypeptide chains are subjected to partial proteolytic digestion in the stacking gel of a second $NaDodSO_4$/polyacrylamide gel with subsequent separation of peptides by electrophoresis into the second gel.

In the present study, a variation of the first-dimension separation was used in which polypeptide chains were separated by isoelectric focusing in 8.5 M urea, as described above, using a gel 1.2 mm thick. Samples of 300–500 μg of hex A or hex B_A were used for the first-dimension separations. After isoelectric focusing, the lane was cut out and incubated for 1 hr in 100 ml of sample buffer (0.065 M Tris·HCl, pH 6.8)/0.1% $NaDodSO_4$ with three changes of buffer to remove ampholines. Subsequently, the lane was embedded horizontally in 1% agarose in the same buffer above a second $NaDodSO_4$/polyacrylamide stacking gel. The second-dimension gel (2 mm thick) had a 6% stacking gel (3 cm × 12 cm) above a 15% running gel (15 cm × 12 cm). Proteolytic digestion was carried out as follows: 75 μg of protease V8 or 300 μg of papain was dissolved in 1 ml of sample buffer in 10% glycerol and overlayed on the stacking gel. Comigration of the proteases and hexosaminidase polypeptides was carried out at 10 mA and interrupted when the tracking dye ran into the middle of the stacking gel. Digestion was allowed to proceed at room temperature for 14 hr for both proteases. Electrophoresis was resumed at 20 mA for 8 hr. Peptides were located by staining with Coomassie blue.

RESULTS

The isoelectric points of the polypeptide chains of reduced and alkylated hexosaminidase were determined by isoelectric focusing in 8.5 M urea. Three independent preparations of hex B, two of hex A, and one of hex B_A were used. All three preparations of hex B (Fig. 1, lanes 1–3), containing only the M_r 25,000 β chains, were resolved into two groups of polypeptides. One group, consisting of a cluster of at least three species designated β_a, showed pI values of pH ≈4.7. The second, more basic group (β_b) showed pI values of pH ≈5.9. Within each group, there were several bands migrating in close proximity to each other with pI values differing by up to 0.2 pH unit. Such microheterogeneity is not unusual and has been reported to result from the presence of slightly differing oligosaccharide side chains (16), deamination of the protein during storage (16), or interaction with cyanates in urea-containing solutions (17).

The β chains of reduced and alkylated hex A were resolved into similar β_a and β_b species (Fig. 1, lanes 4 and 5). An additional band, the M_r 50,000 α chain, migrated with a pI of ≈4.5. The chains also resolved into minor species over a range spanning ≈0.2 pH units.

The isoelectric focusing pattern obtained for the chains of reduced and alkylated hex B_A, formed by the dissociation of the α and β_2 subunits of hex A, was indistinguishable from that of the β_a and β_b species observed for hex B (Fig. 1, lane 6). Hence hex B_A appears to contain β species similar to those of the hex B enzyme. These results indicate that the β_2 subunit of hex A and hex B consists of at least two distinguishable polypeptides that differ in pI by at least 1 unit.

To examine the behavior of the β_a and β_b species by molecular sieving, we used two-dimensional polyacrylamide gel electrophoresis with separation by isoelectric focusing in 8.5 M urea in the first dimension followed by $NaDodSO_4$/polyacrylamide gel electrophoresis in the other. The results of the separation of the polypeptide chains of hex A by using this technique are shown in Fig. 2. The protein species with a pI of 4.5 had an apparent M_r of 50,000, confirming its identification as the α chain. The molecular weights of the proteins denoted β_a and β_b, with pI values of 4.7 and 5.9, respectively, were ≈25,000, as expected for the β chain. There was some indication in the gel that the β_b species had a slightly lower molecular weight than the β_a species.

Having confirmed the identities of the β_a and β_b chains, we next prepared partial proteolytic digests of the polypeptides of hex A and hex B_A to determine whether β_a and β_b differed in primary sequence structure (insufficient true hex B was available for these studies). This was carried out by isoelectric fo-

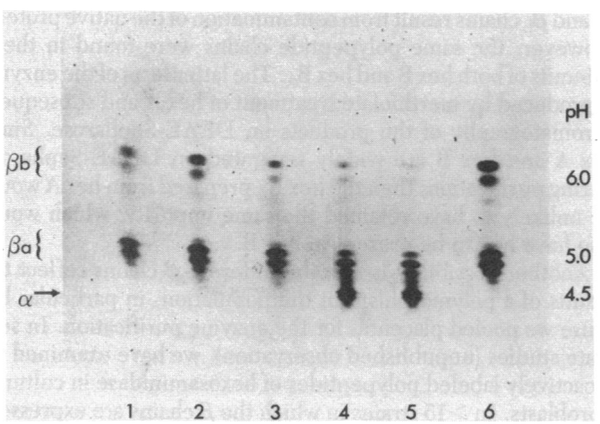

FIG. 1. Isoelectric focusing slab gel of reduced and alkylated hexosaminidase isozymes. Samples (100–200 μg) were applied to each lane at the cathode. Lanes: 1–3, separate preparations of hex B; 4 and 5, separate preparations of hex A; 6, hex B_A.

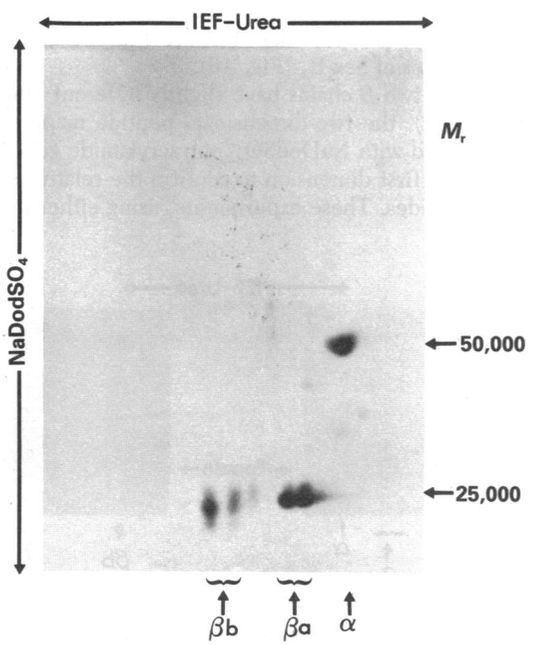

FIG. 2. Two-dimensional polyacrylamide slab gel electrophoresis of reduced and alkylated hex A. Molecular weight separation was by using a 5–15% $NaDodSO_4$ gradient gel and isoelectric focusing (IEF) separation was by using a pH of 3.5–10.0. A sample of 100 μg was applied to the gel.

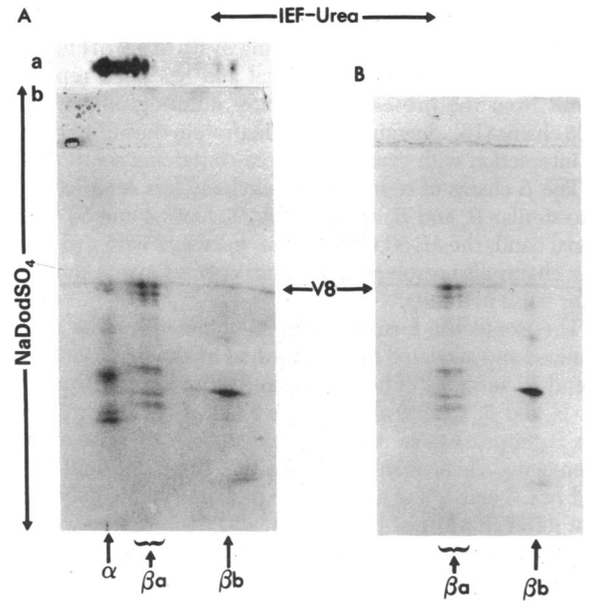

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis of partial protease V8 digests of the polypeptide chains of hexosaminidase. A two-dimensional gel procedure was used in which the polypeptide chains were separated by isoelectric focusing (IEF) in 8.5 M urea in the first dimension (*Aa*) followed by partial protease V8 digestion and peptide separation by $NaDodSO_4$/polyacrylamide gel electrophoresis in the second dimension (*Ab*). (*A*) hex A. (*B*) hex B_A.

cusing of hexosaminidase in 8.5 M urea in the first dimension followed by partial digestion with protease V8 and peptide separation in the second dimension. The peptide profiles obtained for hex A (Fig. 3*A*) showed three distinct peptide patterns, corresponding to the α, β_a, and β_b polypeptides. As expected, the β_a and β_b peptide patterns of hex B_A (Fig. 3*B*) were similar to the corresponding patterns obtained after digestion of hex A. In a similar experiment in which partial papain digestion was used (Fig. 4), distinct peptide patterns were again observed for the α, β_a, and β_b polypeptides of hex A (Fig. 4*A*) and for the β_a and β_b species of hex B_A (Fig. 4*B*).

Because the two β chains have slightly different molecular weights (Fig. 2), the two-dimensional peptide mapping procedure was used with $NaDodSO_4$/polyacrylamide gel electrophoresis in the first dimension to confirm the relative sizes of the β polypeptides. These experiments, using either protease

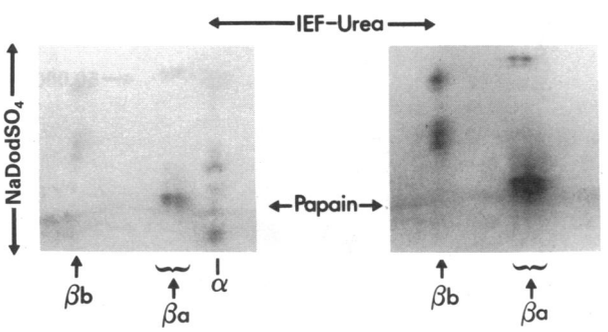

FIG. 4. $NaDodSO_4$/polyacrylamide gel electrophoresis of partial papain digests of the polypeptide chain of hexosaminidase. Polypeptide chains were separated in the horizontal dimension by isoelectric focusing (IEF) in 8.5 M urea. The partial papain digestions were completed in the stacking gel, and the peptides were separated in the vertical dimension by $NaDodSO_4$/polyacrylamide gel electrophoresis. (*A*) hex A. (*B*) hex B_A.

V8 or papain digestion, showed that β_a runs as a slightly higher molecular weight species than β_b (data not shown).

DISCUSSION

We have used a combination of two-dimensional analytical gel electrophoresis and peptide mapping to probe the structure of hexosaminidase. We have confirmed that the α chain behaves as a M_r 50,000 species and is present only in hex A. The results with the β chain were unexpected. Two species of polypeptide chains of $M_r \approx 25{,}000$, described as β_a and β_b, were identified with pI values differing by 1 unit. This β chain pattern was seen consistently in samples from several hexosaminidase preparations and in all the hexosaminidase isoenzyme forms studied (hex A, hex B, and hex B_A). Furthermore, we did not expect to find the pI values of the two groups of bands in hex B to be more acidic than the known pI of the native enzyme (pI = 6.9), although there are earlier reports of pI shifts between native and unfolded proteins (17).

The relationship between the primary structures of the β_a and β_b polypeptide chains was examined by peptide mapping. Distinct peptide patterns were obtained for the α, β_a, and β_b chains after partial digestion with either protease V8 or papain. Similar results were obtained, differing only in the extent of digestion, whether $NaDodSO_4$/polyacrylamide gel electrophoresis or isoelectric focusing in urea was used in the first dimension or, in the case of the β chains, whether they came from hex B or hex B_A. These results indicate that the β_a and β_b species have different amino acid sequences.

Further support for the existence of two β chains comes from our earlier study of two-dimensional peptide maps of a tryptic digest of hexosaminidase (8). The digest showed ≈48 separate peptide spots. On amino acid analysis, hex B contained 100 arginine/lysine residues in the intact enzyme (unpublished data; refs. 1, 2, and 18). If hex B is the homopolymer of four identical β chains (i.e., $2\beta_2$), then each would have been expected to have 25 arginine/lysine residues and a maximum of 26 tryptic peptides would have been produced. If hex B contained two nonidentical polypeptide chains, however, ≈50 resolvable peptides could have been generated. Differences in oligosaccharide side chain or other posttranslational modification could not account for the results of peptide mapping since there are, at most, one or two oligosaccharide side chains per β chain (19). Therefore, we conclude that the β_a and β_b species have different amino acid sequences.

One could speculate that the different peptide patterns of the β_a and β_b chains result from contamination of the native protein. However, the same polypeptide chains were found in the β subunits of both hex B and hex B_A. The latter form of the enzyme is produced by merthiolate treatment of hex A and subsequent chromatography of the products on DEAE-Sepharose. Since hex A and hex B are widely separated on DEAE-Sepharose during purification, then the hex B_A prepared from hex A would be unlikely to have retained the same impurity, which would also have had to be present in hex B.

Another possibility is that the different β chains reflect the results of a polymorphism in the population, in particular because we pooled placentas for the enzyme purification. In separate studies (unpublished observation), we have examined radioactively labeled polypeptides of hexosaminidase in cultured fibroblasts. In >15 strains in which the β chains are expressed, both the β_a and the β_b chains were synthesized in all cases. Thus far, we have not identified any fibroblast strains in which one β chain but not the other is expressed.

Several lines of evidence indicate that hex B is the product of a single gene. The enzyme has been mapped to a single chro-

mosome, 5 (7), although the experiments could not have distinguished between one or more than one gene on that chromosome. Second, fibroblasts from patients having Sandhoff disease fail to produce any mature β chains or β precursor (5). Finally, several fibroblast strains from patients having Sandhoff disease have failed to complement in heterokaryons (20), even in the case of a fusion between the infantile and a rare juvenile form of the disease (21).

How do these results fit with the identification of the β_a and β_b polypeptide chains? In fibroblast extracts precipitated with specific hexosaminidase antisera, Hasilik and Neufeld (5) have identified a M_r 60,000 polypeptide chain that is absent in Sandhoff cells and immunochemically related to the β chain. This larger polypeptide appears in pulse–chase experiments to be a precursor of the β chain. The existence of such a precursor would provide an attractive explanation for the presence of distinct M_r 25,000 β_a and β_b species. It is possible that the M_r 60,000 polypeptide chain is clipped in approximately equal parts and then otherwise processed to form the final β_a and β_b species. Thus, β_a and β_b could be encoded, for example, by the left and right side of a single gene. This conjecture suggests that hex A and hex B contain both the β_a and β_b species to give $\alpha\beta_a\beta_b$ for hex A and $2(\beta_a\beta_b)$ for hex B.

There are precedents for the processing of a gene product to give two or more functional polypeptide chains apparently originating by a proteolytic clipping of the parent chain. These include the successive clipping of virion proteins of retroviruses to generate smaller subunits (22) and the proteolytic processing of preproinsulin and proinsulin to yield the two polypeptide chains of mature insulin (23).

Such a one gene–two polypeptide chains explanation would be consistent with the involvement of a single chromosome and with the absence of complementation in Sandhoff disease. Mutations in the β chain gene could result in the absence of the mature form of either the β_a or β_b species in immunoprecipitates of fibroblast extracts.

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