Identification of the binding and activating sites of the sphingolipid activator protein, saposin C, with glucocerebrosidase

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Abstract

Saposin C is a sphingolipid activator protein of 8.5 kDa that activates lysosomal glucocerebrosidase. Previously, we synthesized and characterized a synthetic full-length human saposin C protein that displays 85% of the activity of the native saposin C. In this study we use shorter synthetic peptides derived from the saposin C sequence to map binding and activation sites. By determining the activity and kinetic constant (K_{act}) values of these peptides, we have identified two functional domains, each comprising a binding site adjacent to or partially overlapping with an activation site. Domains 1 and 2 are located within amino acid positions 6–34 and 41–60, respectively. The activation sites span residues 27–34 and 41–49, whereas binding sites encompass residues 6–27 and 45–60. Peptides containing the sequences of either domain displayed 90% of the activity of the full-length synthetic saposin C. Domain 2, however, bound to glucocerebrosidase by at least an order of magnitude more strongly than domain 1. Binding sites within these domains contain sequences that are excellent candidates for forming amphipathic helical structures. Competition assays demonstrated that the binding of one domain to glucocerebrosidase prevents binding of the other domain, and that saposin A and saposin C bind to the same sites on glucocerebrosidase. A model predicting a saposin C:glucocerebrosidase complex with a stoichiometry of 4:2, respectively, is presented.

Keywords: Ceredase™; deglycosylation; enzyme activation; Gaucher disease; glucocerebrosidase; saposin A; saposin C; sphingolipid activator proteins; synthetic peptides

Human glucocerebrosidase, lysosomal β -glucosyl-*N*-acylsphingosine glucosylhydrolase (E.C. 3.2.1.45), hydrolyzes the β -glycosidic linkage of ubiquitous naturally occurring neutral glycosphingolipids in the class of compounds known as cerebroside. The etiology of a group of inherited lysosomal storage

diseases, known as Gaucher disease, has been ascribed to a deficiency of the enzyme (Brady et al., 1965; Patrick, 1965), which leads to the accumulation of its substrates in the lysosomes of cells of the reticuloendothelial system (Radin, 1984; Barranger & Ginns, 1989). This enzyme has recently received considerable attention on two fronts. (1) Enzymatic processing of the four complex carbohydrate chains of glucocerebrosidase back to the mannose core (Furbish et al., 1981) results in an active enzyme that is specifically targeted to the lysosomes of liver Kupffer cells (Murray & Jin, 1995). This form of the enzyme, when administered to Gaucher patients, results in a dramatic decrease in the severity of their disease. The macrophage-targeted enzyme is marketed for clinical use under the name of Ceredase™ by Genzyme (Cambridge, Massachusetts). (2) The native enzyme is also a leading candidate for gene replacement therapy. Long-term

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Abbreviations: *t*-boc, tert-butyloxycarbonyl; SAP, sphingolipid activator protein; HF, hydrofluoric acid; TFA, trifluoroacetic acid; K_{act} , concentration of activator protein that yields 50% maximal activity.

expression in cultured mouse bone marrow stem cells has been achieved (Ohashi et al., 1992; Bahnson et al., 1994; Nimgaonkar et al., 1994).

The highest level of catalytic activity observed for glucocerebrosidase, *in vitro*, is dependent on the presence of two small activator proteins, saposin C and saposin A (Fig. 1; Ho et al., 1973; Berent & Radin, 1981) as well as acidic phospholipids (Ho & Light, 1973; Dale et al., 1976). The negative charge of the phospholipid appears to be essential (Basu et al., 1985) and phospholipids containing short or polyunsaturated fatty acids are the most effective activators (Basu & Glew, 1984; Basu et al., 1984). Acidic phospholipids and saposin C (or saposin A) display an additive effect on the activation of glucocerebrosidase, suggesting that they bind at different sites (Basu & Glew, 1984). No additive effect between saposin A and saposin C is observed, indicating that these two proteins may bind to the same site(s) on glucocerebrosidase (Morimoto et al., 1990).

Saposin C, discovered by Ho and O'Brien (1971), is one of four different saposins (A–D) generated by partial proteolysis of a common precursor, prosaposin (Fujibayashi & Wenger, 1986). These mature saposins are required for the hydrolysis of sphingolipids by specific lysosomal hydrolases (reviewed by O'Brien & Kishimoto, 1991).

It has been suggested by others that a deficiency of saposin C can cause disease with the Gaucher-like phenotype: three patients with normal *in vitro* glucocerebrosidase activity but with an absence of saposin C have recently been reported (Christomanou et al., 1986, 1989; Harzer et al., 1989). All three cases presented neurologic disorders and were fatal. In one patient, a point mutation in the gene encoding prosaposin results in a Cys to Phe substitution in the saposin C domain and has been postulated to give rise to an unstable mature protein (Schnabel et al., 1991). In the most severe case, the patient displayed combined sphingolipidoses (Paton et al., 1992) and an absence of precursor protein as a result of a point mutation in the initiation codon of the prosaposin gene (Schnabel et al., 1992). These results, if verified, would indicate a true physiological role for saposin C.

A full understanding of the interaction between glucocerebrosidase and saposin C would include identifying the location and nature of the interactive sites. This would be an important step

toward delineating the mechanism of activation at the molecular level.

Previously, we had synthesized and characterized a folded full-length saposin C molecule that displayed 85% of the native biological activity (Weiler et al., 1993). In this report we map and characterize the binding and activating sites on saposin C and use competition assays in preliminary experiments to determine the nature of the complementary sites on glucocerebrosidase. One peptide that binds tightly but with limited activating capabilities was used in conjunction with native saposin A and C to ascertain whether or not saposins A and C share a common binding site.

Results

Mapping of binding and activation sites

Synthetic peptides derived from the saposin C sequence (Fig. 2) were tested for their ability to bind and activate glucocerebrosidase-catalyzed hydrolysis of the fluorescent substrate analog 4-methylumbelliferyl- β -D-glucoside. These experiments presented in this study were carried out in the absence of other activators such as phosphatidylserine. In the few experiments in which other stimulators were present we found activation to be additive with phosphatidylserine and nonadditive with bile salts such as sodium taurocholate. Much of the data are reported as the concentration required to achieve half-maximal activation and represent an approximation of the binding constant. We use the term K_{act} to define this value, but it should be noted that it could also be termed Act_{50} . Previously we had shown that a full-length synthetic saposin C stimulated glucocerebrosidase by 6.0-fold compared with a value of 7.1-fold for native saposin C purified from human spleen at activator protein saturating concentrations of 10 mM. The K_{act} value for synthetic saposin C was threefold lower than that measured for native saposin C suggesting that the synthetic molecule bound to glucocerebrosidase somewhat more tightly. Analysis of the shorter peptide sequences revealed two domains (Fig. 1) of saposin C capable of both binding and activating glucocerebrosidase. The first domain was mapped to residues 41–68. This peptide displayed 82% of the activity (maximal stimulation) of the full-length molecule

Fig. 1. Amino acid sequences of human saposin C (upper line) and saposin A (lower line). Conserved cysteines, prolines, and glycosylation sites, at Asn 22, are shown in bold print. Location of mapped functional domains postulated for only saposin C are also shown.

Fig. 2. Deletional analysis of synthetic saposin C. Short peptides containing saposin C sequences were tested for activity and compared to an 82-residue synthetic saposin C molecule (top line) previously synthesized and characterized (Weiler et al., 1993). Maximal activation refers to the increase (fold) in hydrolytic rate of glucocerebrosidase in the presence of saturating concentrations of the peptide. K_{act} is the concentration of the peptide required to increase the hydrolytic rate of glucocerebrosidase by 50% of the peptide's maximal activation value. Peptides 21-40 and 13-40 were weak binders and could not saturate glucocerebrosidase at the concentrations tested (up to 5 mM). Kinetic values for these peptides, shown in parentheses, are estimates.

with a K_{act} value that was at least an order of magnitude greater. Successive deletions of sequences from the N-terminal end of either peptide 41-68 and 41-82 resulted in a marked drop in maximal stimulation values, indicating the existence of an activating site within the region 41-49.

Successive deletion of sequences C-terminal to the activating site weakened the binding as demonstrated by the K_{act} values for peptide 41-82, 41-68, 41-60, and finally 41-55, which showed no activity presumably due to a complete lack of binding (data not shown). Surprisingly, these deletions also had the effect of increasing bioactivities of the peptides.

A second binding and activating domain was mapped near the N-terminal end of saposin C. Peptides 1-27 and 6-34 have similar K_{act} values, but their activity values are 20 and 92% of the full-length molecule, respectively, indicating that an activating site resides at residues 27-34. This is supported by the high activity values of other peptides containing this site. Peptide 6-34 is able to activate glucocerebrosidase to approximately the same degree but its binding is an order of magnitude weaker when

compared to a peptide of equivalent length (peptide 41-68) in the C-terminal domain.

Competition assays

Assays were performed to determine whether a peptide containing only the binding site of one domain could disrupt the binding and activation of glucocerebrosidase by (1) full-length synthetic saposin C or peptides containing both binding and activation sites derived from one of the two domains (Fig. 3), as well as (2) native saposin C and native saposin A (Fig. 4). In each case, a number of different concentrations of activating peptide or protein were chosen to be high enough to activate glucocerebrosidase by at least fourfold and low enough for effective inhibition by binding but nonactivating peptides (optimization experiments not shown). In all cases, the optimized concentrations were below saturating levels.

From these experiments, it was found that optimal concentrations of the activating peptides, as reported in Figures 3 and

Fig. 3. Competition of "high binding/low activation" peptides with high activating peptides. A preformed complex of glucocerebrosidase with synthetic saposin C ($5 \mu\text{M}$) was challenged with either peptide 49-82 (A) or peptide 1-27 (B). A complex of glucocerebrosidase with peptide 41-60 ($30 \mu\text{M}$) was challenged with either peptide 49-82 (C) or peptide 1-27 (D). Finally, a complex of glucocerebrosidase with peptide 6-34 ($200 \mu\text{M}$, circles; or $500 \mu\text{M}$, squares) was challenged with either peptide 49-82 (E) or peptide 1-27 (F).

Fig. 4. Competition of peptide 49-82 with native saposin proteins. Pre-formed complexes of glucocerebrosidase with native saposin C (5 μ M, **A**) or native saposin A (15 μ M, **B**) were challenged with peptide 49-82.

4, did not display a consistent relationship with the peptide versus K_{act} value, suggesting other factors, such as the stability of the preformed complex, may play a role in the response of the complex to the competitively inhibiting peptides. It should also be noted that during the optimization of these reactions, higher concentrations than reported of the activating peptides in the preformed complex were tested in conjunction with increasing quantities of the competing/inhibitory peptides. Near linear responses were observed throughout the concentration ranges indicating that the loss of glucocerebrosidase activity reflected loss of activation and not the quenching of fluorescence by high concentrations of peptide. Peptides 1-27 (part of domain 1) and 49-82 (part of domain 2) were chosen as suitable competitors as they displayed significant binding to glucocerebrosidase (K_{act} = 140 μ M and 6 μ M, respectively) but were capable of only weakly activating the enzyme (maximal stimulation = 2.0- and 2.1-fold, respectively). Both peptides 49-82 and 1-27 reduced the activation of glucocerebrosidase by full-length saposin C to levels approaching twofold (Fig. 3A,B), indicating that a binding site from either domain can completely disrupt a complex formed between saposin C and glucocerebrosidase. This suggests that

the two domains may bind to the same site on glucocerebrosidase or to two sites that are in close proximity to one another. To support this hypothesis further, we challenged a preformed complex of peptide 41-60 and glucocerebrosidase with peptide 1-27 and observed successful competition (Fig. 3D). In the reciprocal experiment, peptide 49-82 reduced the level of activation of glucocerebrosidase by peptide 6-34 (Fig. 3E). More analyses have to be carried out in order to determine the nature of the competition.

Peptide 1-27, in a dose-dependent manner, reduced the level of activation of glucocerebrosidase by peptide 6-34 (Fig. 3F). A similar result was observed when peptide 49-82 was used to challenge an activated complex formed between glucocerebrosidase and peptide 41-60 (Fig. 3C). In both cases, the level of activation either approached or dropped to twofold at saturating concentrations of the competing peptides (the level expected to be generated by the competitive inhibitors alone). The high initial activity observed for the 41-60 peptide (higher than that predicted based on its K_{act} of 30 μ M) reflects the variation observed for the different lot of the enzyme used in this one experiment. The apparent fivefold increase should not be viewed as an absolute value because it represents only a fraction of the potential activation observed in that set of experiments. The results presented in Figure 3 support the finding (by deletional analysis) that the binding and activation sites within a domain are not contained in the same amino acid sequences, but are either adjacent or partially overlapping.

Activation of glucocerebrosidase by either native saposin C or native saposin A is also reduced to twofold (the value expected for the weakly activating peptide) when a preformed complex of either of the saposins with the enzyme is challenged with peptide 49-82 (Fig. 4A,B, respectively). The differences in the concentrations tested reflect the differences in concentration where these activators reached levels of maximal stimulation of glucocerebrosidase; for native saposins C and A we measured these concentrations to be approximately 10 and 25 micromolar, respectively. The result that the same peptide inhibited either saposin (C or A) indicates that both saposins bind to the same site(s) on glucocerebrosidase.

Discussion

Characterization of the functional domains

Structural studies of saposin C revealed a high α -helical content as determined by CD (O'Brien & Kishimoto, 1991). NMR experiments have indicated the presence of a rigid hydrophobic core (Sheh et al., 1985). Recently, Stevens et al. (1993) mapped the disulfide bridges of the 79-residue porcine saposin B (also known as cerebroside sulfate activator or SAP-1). Based on their data, they proposed a structural model of saposin B consisting of four amphipathic helices stabilized by interhelical disulfide bonds. Peptides containing domain 1 and domain 2 of saposin C both displayed approximately 90% of the activity of full-length synthetic saposin C. Domain 2, however, bound glucocerebrosidase by an order of magnitude tighter than domain 1. By substituting amino acid sequences derived from domain 2, into helical wheels, we have located a sequence between amino acids 45 and 62 that is an excellent candidate for an amphipathic helix (Fig. 5A). The hydrophilic face contained only acidic and hydroxyl residues with all acidic residues residing in the N-ter-

Fig. 5. Hypothetical helix representations of the putative binding site of domain 2. **A:** Helical wheel demonstrating the amphipathic nature of the helix. **B:** Longitudinal view of the helix, showing the distribution of the negatively charged and hydroxyl-containing residues.

minimal half of the sequence (Fig. 5B). Interestingly, one of the proposed helices in saposin B (Stevens et al., 1993), located between amino acid positions 43 and 59, almost superimposes (with respect to the six conserved cysteine residues) on the putative amphipathic helix (Fig. 5) that we have proposed as the binding site for saposin C.

A peptide containing this sequence alone displayed good binding ($K_{act} = 55 \mu M$) and activation (maximal stimulation = 4.2-fold). The activation values, however, were significantly lower than the overlapping peptide at 41–60 (maximal stimulation = 5.6-fold), indicating that part of the activation site of domain 2 lies outside this putative helix. Addition of any of the amino acids 41–44 into the helical wheel containing residues 45–62 disrupted the amphipathic nature of this helix. We therefore propose that the binding site of domain 2 is an amphipathic helix and that it contains part of the activation site. Furthermore, we propose that the improved binding observed for peptides containing additional sequences adjacent to the helix (K_{act} for peptide 45–82 = $3.6 \mu M$) is a result of stabilization of this helix. Similarly within domain 1, a putative amphipathic helix can be assigned to amino acid positions 3–21. This probably contributes to the binding of peptides 1–27 and 6–34. A sharp drop in binding of the peptides was observed with losses of sequences in the region between 6 and 21. As a further note, domain 1 has been shown to promote neurite outgrowth in culture suggesting a separate function for this portion of the molecule (O'Brien et al., 1995).

From the deletion analyses with synthetic peptides, we have shown that residue Asn 22, which is the glycosylation site of na-

tive saposin C, is located within the binding site (but outside the activation site) of domain 1. To determine what effect the carbohydrate moiety had on binding, we accurately measured K_{act} values of native, deglycosylated native, and synthetic saposin C. K_{act} values for both deglycosylated native and synthetic saposin C were $1 \mu M$ compared with native saposin, which was $3 \mu M$ (data not shown). No loss of activity was observed upon deglycosylation. These results suggest that the difference in binding between the synthetic preparation and native saposin C is due to the presence of carbohydrate moiety in the binding site and not due to differences in the polypeptide backbone structures.

Saposin C-glucocerebrosidase complex

Results of previous kinetic and physical studies suggest that the complex formed between glucocerebrosidase and saposin C does not display simple 1:1 stoichiometry. Ho and Rigby (1975) demonstrated a biphasic nature in enzyme activity plots in the range of 0.1–10-fold of activator to enzyme. Based on their experimental data, they deduced a model describing a two-step assembly of an activated complex containing two molecules of saposin C and one molecule of glucocerebrosidase. The model predicts that the intermediate complex (1:1) is rapidly formed at low saposin C concentrations and displays only 28% of the activity of the fully activated complex (2:1), which required higher concentrations of saposin C for formation. Prentice et al. (1985) showed, by sucrose density gradient ultracentrifugation, that preincubation of rat liver glucocerebrosidase with phosphatidylserine and saposin C resulted in a complete shift in the enzyme from a

57-kDa to a 188-kDa form. The high molecular weight form was highly activated and exogenous activators, such as sodium taurocholate, had no effect on the activity of the complex. The lower molecular weight form, however, required these activators for activity. Using gel-permeation HPLC, Aerts et al. (1987) detected and separated both nonactivated (60 kDa) and fully activated (200 kDa) forms of glucocerebrosidase from extracts of human spleen. The high molecular weight form contained material that cross-reacted with both their anti-glucocerebrosidase and anti-saposin C antibodies.

From these observations, together with data from our mapping, competition, and deglycosylation experiments, we propose a model describing the assembly of an activated complex. Our model makes the assumption that a complex of 180–200 kDa is a prerequisite for maximal activity of glucocerebrosidase and would have to include at least two glucocerebrosidase molecules. In determining which interactions are permissible, we have eliminated certain combinations using our mapping and competition data. Firstly, because a short peptide containing a single binding site can mimic the effect of the full-length molecule, we propose that saposin C does not bind two glucocerebrosidase molecules through each of its domains. By the same reasoning, two saposin C molecules cannot interact with each other. Thus, glucocerebrosidase molecules in the activated complex would have to interact directly with each other. We propose that a dimerization of glucocerebrosidase is brought about by a change in the enzyme initiated by saposin C or saposin C in conjunction with acidic phospholipids.

Because peptides containing either domain 1 or domain 2 bind the same or equivalent sites on glucocerebrosidase, we believe that full-length saposin C binds glucocerebrosidase predominantly through domain 2, because peptides containing this domain display K_{act} values of an order of magnitude lower than peptides containing domain 1. This difference may be more pronounced in vivo because the presence of the carbohydrate moiety in native saposin C appears to increase K_{act} for the full-length molecule when compared to synthetic or deglycosylated saposin C. These observations, together with the fact that only one domain is required for full activation, suggests that domain 1 may not play a significant role in complex formation and activation.

A two-step model involving saposin C binding 1:1 to glucocerebrosidase, followed by dimerization of this complex yielding a 2:2 stoichiometry, would not fit the data reported by Ho and Rigby (1975) because the biphasic nature of the activation of glucocerebrosidase by saposin C was shown to be dependent on the activator's concentration. Furthermore, this activated complex would have a molecular weight of only 150–160 kDa. Although present models predict one binding site on glucocerebrosidase for acidic phospholipids, it still remains unclear how many lipids bind per glucocerebrosidase molecule. Therefore, we cannot completely rule out the possibility that these activating lipids bind to glucocerebrosidase, in the complex, in sufficient quantities to account for the molecular weight discrepancy (150–160 kDa versus 180–200 kDa). Nevertheless, we propose that two molecules of saposin C bind per glucocerebrosidase molecule. The two sites of glucocerebrosidase would be equivalent with respect to the sequences that they recognize on saposin C (or saposin A), but different in their binding affinities. Subsequent dimerization of this intermediate 2:1 complex would give the fully activated 4:2 complex. Such a complex would be

consistent with previous kinetic observations, our mapping and competition studies, and the requirement for the fully activated complex to be between 180 kDa and 200 kDa (molecular weight of glucocerebrosidase, 60–67 kDa; saposin C, 8.5 kDa).

Saposin A and saposin C share a common binding site

Our competition studies suggest that saposin C and saposin A bind to the same site(s) on glucocerebrosidase, probably through similar sites of the saposins. These data support our previous observations in which no additive nor synergistic effect was observed using mixtures of saposin C and saposin A to activate glucocerebrosidase (Morimoto et al., 1990). By contrast, kinetic studies with saposins A and C conducted by Fabbro and Grabowski (1991) have led them to postulate that these proteins bind different sites on glucocerebrosidase. The sequence identity between saposins A and C is 30%. This increases to 45%, comparing sequences only within domain 2. Furthermore, most of the changes are conservative within this region and maintain a putative amphipathic helical nature within the equivalent region of saposin A. The high conservation of this sequence further supports our hypothesis that saposin A and saposin C bind to the same site(s) on glucocerebrosidase through equivalent sites on the activator proteins.

Identification of the saposin C binding sites may prove useful in designing improved strategies for treating Gaucher disease. Presently, enzyme replacement therapy has proved successful but extremely expensive, due to the large quantities of glucocerebrosidase required for clinical effect (Beutler, 1992). Small peptides containing the biologically active region(s) of saposin C may have potential therapeutic use administered either directly with or crosslinked to glucocerebrosidase.

Materials and methods

Materials

Human glucocerebrosidase was purified from placenta by the method of Aerts et al. (1986). Native human saposin C was prepared from the spleen of a Gaucher patient by the method of Morimoto et al. (1989). Full-length synthetic saposin C was previously synthesized (Weiler et al., 1993). *N*-glycanase (*N*-glycosidase F) was obtained from Boehringer Mannheim. All N^{α} -*t*-boc amino acids were obtained from Peninsula Laboratories, except *t*-boc-(dinitrophenyl) histidine, which was from Fluka. The resins and *t*-boc-[amino acid]-OCH₂-PAM were obtained from Applied Biosystems Inc. β -Mercaptoethanol, *p*-cresol, and *p*-thiocresol were obtained from Sigma. Anhydrous HF was obtained from Mattheson. Anhydrous diethyl ether was obtained from Mallinckrodt. Diisopropylethylamine and TFA were obtained from Aldrich. Dimethylformamide (stored over molecular sieve), methanol, dichloromethane, acetonitrile, and isopropanol were obtained from Burdick and Jackson and were all HPLC grade.

Peptide synthesis

Peptides containing sequences derived from saposin C (Figs. 1, 2) were synthesized using PAM resins and an automated solid-

phase protocol, based on the principles outlined by Merrifield (1963), on an Applied Biosystems Model 430 peptide synthesizer. In order to optimize the solvation of the resin and coupling efficiencies, varying levels of dichloromethane and dimethylformamide containing *N*-methyl pyrrolidone were tested. Multiple couplings of protected amino acid residues at each step were also employed to increase the coupling efficiencies. Quantitative ninhydrin assays (Sarin et al., 1981) of representative couplings revealed that the average coupling efficiency was approximately 99.5%. After assembly, polypeptides containing histidine residues in their sequence were treated twice for 30 min at 25 °C with 15 mL of 10% diisopropylethylamine/20% β -mercaptoethanol in dimethylformamide to remove the dinitrophenol protecting group. The N^{α} -*t*-boc groups of all peptides were subsequently removed with 65% TFA for 10 min at 25 °C. Each polypeptide/resin was dried and immediately treated with anhydrous HF. Cleavage and full deprotection of the polypeptide was carried out in the presence of scavenger (*p*-cresol), reducing agent (*p*-thiocresol), and precondensed anhydrous HF for 30 min at -10 °C and 60 min at 0 °C employing an Immuno-Dynamics HF cleavage apparatus. The condensed HF was removed at the end of the reaction, and the cleavage reaction mixture was washed with 100 mL of cold anhydrous diethyl ether. The resultant precipitate, which contained the cleaved peptide and vinylbenzene resin, were collected and dried in vacuo over KOH.

All peptides were purified in the same manner. Because some peptides were insoluble at pH values below 7.5 and had a tendency to aggregate, a modified purification scheme was employed. Peptides were dissolved in 0.2 M ammonium bicarbonate (pH 8.5), filtered to remove the resin, then applied to a Sephadex G-75 column (bed volume = 20 mL) equilibrated and run in 50 mM ammonium bicarbonate (pH 8.5) containing 2.5% acetonitrile and 2.5% isopropanol. For each peptide the front half of the first eluted peak was retained and dried in vacuo. The sequences of the peptides were verified by N-terminal sequencing using standard pulsed liquid methods on an Applied Biosystems Model 477A protein sequencer.

Deglycosylation of saposin C

Native saposin C (200 μ g) was incubated in 0.4 M $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 8.6) containing 0.2% SDS and 0.4% β -mercaptoethanol. After heating the solution at 100 °C for 3 min, the solution was cooled and 80 μ L of 6.25% NP-40 and 80 μ L of 50 mM phenanthroline was added. N-glycanase (5 U in 20 μ L) was added and the mixture incubated at 37 °C for 18 h. The reaction was monitored by SDS-PAGE on a precast 16% tricine minigel (100 $\times$ 1 mm) obtained from Novex. Protein bands were visualized using a BioRad silver stain kit. Deglycosylated saposin C was purified by reversed-phase HPLC on a PLRP-S reverse-phase column from Polymer Laboratories (300 Å, 8 μ m particle size, 150 $\times$ 4.6 mm). The column was equilibrated with Buffer A (2.5% acetonitrile; 2.5% isopropanol; 95% 50 mM ammonium bicarbonate, pH 8.5). After injection of the reaction mixture, the initial conditions were maintained for 5 min, followed by a linear gradient of 0–100% Buffer B (40% acetonitrile; 40% isopropanol; 20% 50 mM ammonium bicarbonate, pH 8.5) over 90 min. The column was run at a flow rate of 0.8 mL/min and the optical density of the eluent was measured at 225 nm.

Glucocerebrosidase activation assay

Increasing amounts of synthetic peptides were preincubated with 15 U of purified glucocerebrosidase (human placental) in 50 μ L of 0.1 M sodium acetate (pH 5.0) containing 0.1% Triton X-100 and 1 mg/mL bovine serum albumin for 15 min at room temperature. This was followed by the addition of the substrate analog, 4-methylumbelliferyl- β -D-glucoside, to a final concentration of 5 mM in 200 μ L containing 0.1 M sodium acetate (pH 5.0) and 0.1% Triton X-100. The reaction was carried out at 37 °C for 30 min and terminated by the addition of 3.8 mL of 0.17 M glycine carbonate buffer (pH 10.4). An Hitachi fluorometer model F-2000 was used to monitor the increase in fluorescence resulting from the generation of the highly fluorescent product, 4-methylumbelliferone. In order to determine the extent of binding and/or activating capacity of a peptide, two kinetic parameters were measured for each peptide: (1) maximal stimulation, the increase in the hydrolytic rate of glucocerebrosidase in the presence of saturating levels of peptides; and (2) K_{act} . The values calculated for the latter term were used only as an approximation of the relative strength of binding of the saposin C peptides to the enzyme.

Competition assays

Preformed activated complexes of glucocerebrosidase with the synthetic activating peptides, full-length saposin C (concentration in final reaction mixture = 5 μ M), peptide 41–60 (30 μ M), and peptide 6–34 (200 μ M or 500 μ M) were challenged with increasing concentrations of either peptide 49–82 or peptide 1–27: Activating peptides were diluted in water to contain the correct amount in 1 μ L. This was added directly to 1.8 U of glucocerebrosidase (specific activity: 9×10^5 U/mg) in 2.5 μ L of 0.1 M sodium acetate (pH 5.0) containing 0.1% Triton X-100 and 1 mg/mL bovine serum albumin. After preincubation at room temperature for 15 min, the competing peptides (49–82 and 1–27) were added to the reaction mixture. The volumes of the reaction mixtures were brought up to 12.5 μ L and the overall buffer concentrations brought to 0.1 M sodium acetate (pH 5.0), 0.1% Triton X-100, and 1 mg/mL bovine serum albumin with a 10 $\times$ buffer stock solution and water. After 15 min incubation, aliquots of 37.5 μ L of 6.67 mM 4-methylumbelliferyl- β -D-glucoside in 0.1 M sodium acetate (pH 5.0) with 0.1% Triton X-100 were added to the reaction mixtures and the hydrolysis allowed to proceed at 37 °C for 30 min. The reaction was terminated with 0.95 mL of a 0.17 M glycine carbonate buffer.

Complexes formed between glucocerebrosidase and either native saposin C (5 μ M) or native saposin A (15 μ M) were challenged with increasing concentrations of peptide 49–82 using an identical protocol to the one given above. Preliminary experiments of native saposins C and A with glucocerebrosidase showed that these activators reached levels of maximal stimulation at approximately 10 and 25 μ M, respectively.

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