Modulation of circulatory residence of recombinant acetylcholinesterase through biochemical or genetic manipulation of sialylation levels

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Sialylation of N-glycans associated with recombinant human acetylcholinesterase (rHuAChE) has a central role in determining its circulatory clearance rate. Human embryonal kidney 293 (HEK-293) cells, which are widely used for the expression of recombinant proteins, seem to be limited in their ability to sialylate overexpressed rHuAChE. High-resolution N-glycan structural analysis, by gel permeation, HPLC anion-exchange chromatography and high-pH anion-exchange chromatography (HPAEC), revealed that the N-glycans associated with rHuAChE produced in HEK-293 cells belong mainly to the complex-biantennary class and are only partly sialylated, with approx. 60 % of the glycans being monosialylated. This partial sialylation characterizes rHuAChE produced by cells selected for high-level expression of the recombinant protein. In low-level producer lines, the enzyme exhibits a higher sialic acid content, suggesting that undersialylation of rHuAChE in high-level producer lines stems from a limited endogenous glycosyltransferase activity. To improve sialylation in HEK-293 cells, rat liver β-galactoside α-2,6-sialyltransferase cDNA was stably transfected into cells expressing high levels of rHuAChE. rHuAChE produced by the modified cells displayed a significantly higher proportion of fully sialylated glycans as shown by sialic acid incorporation assays, direct measurement of sialic acid, and HPAEC glycan profiling. Genetically modified sialylated rHuAChE exhibited increased circulatory retention (the slow-phase half-life, $t_{\frac{1}{2}}\beta$, was 130 min, compared with 80 min for the undersialylated enzyme). Interestingly, the same increase in circulatory residence was observed when rHuAChE was subjected to extensive sialylation *in vitro*. The engineered HEK-293 cells in which the glycosylation machinery was modified might represent a valuable tool for the high level of expression of recombinant glycoproteins whose sialic acid content is important for their function or for pharmacokinetic behaviour.

INTRODUCTION

Acetylcholinesterases (AChEs, EC 3.1.1.7), as well as the related butyrylcholinesterases, display tissue-specific variation in their structures. Assembly of catalytic and structural subunits results in the formation of several classes of membrane-bound and soluble cholinesterase forms [1]. Serum-derived cholinesterases represent a specific group of soluble homo-oligomeric forms that reside in the circulation for extended periods. When human serum butyrylcholinesterase or fetal bovine serum AChE are administered exogenously to experimental animals, they display high serum residence and are cleared from the circulation after several hours [2–5]. This high serum residence could not be reproduced when native or recombinant AChEs (rAChEs) from other sources were examined [4,6,7]. Cholinesterases therefore represent a group of closely related proteins displaying pharmacokinetic variations and could provide a good experimental system for the analysis of molecular factors involved in the clearance of proteins from the circulation.

Differences in clearance rates of proteins might originate from variations in their primary sequences or in post-translational modifications. We have explored these possibilities in the context of the AChE family and found recently that the difference between the sequences of bovine AChE and human AChE cannot explain the difference between the clearance profiles of native bovine serum-derived AChE and recombinant human acetylcholinesterase (rHuAChE) produced in human embryonal kidney 293 (HEK-293) cells [7]. In contrast, our studies with various glycosylation mutants of rHuAChE indicated that N-glycosylation has a central role in determining the circulatory residence in cholinesterases [4]. Furthermore the efficiency of N-glycan sialylation was shown to determine the rate of clearance of the AChEs; an inverse linear relationship was shown to exist between the number of asialylated glycan termini associated with the enzyme and its rate of clearance. The effect of terminal glycosylation, including sialylation, on clearance rates was also demonstrated in a variety of other proteins [8]. Therefore the distribution and abundance of various terminal glycosyltransferases [9,10] in cells used to produce native or rAChEs could have a key role in their pharmacokinetic profiles. This is of importance in particular for the generation of recombinant proteins whose potential therapeutic value has been impeded by their fast clearance rate [11,12].

Cholinesterases have been assigned a therapeutic value by virtue of their affinity or scavenging ability for organophosphate poisons [3,13–16]. Recombinant human AChE, produced in large amounts in stably transfected cells [17,18] could be a suitable source for this bioscavenger. We have shown in the past that it is indeed possible to improve the bioscavenging performance of recombinant human AChE by site-directed mutagenesis. Mutagenesis either enhanced the enzyme affinity for organophosphate compounds or prevented the aging process [19,20].

Here we address the role of sialylation in determining the

Abbreviations used: α2,6ST, β-galactoside α-2,6-sialyltransferase; AChE, acetylcholinesterase; HEK-293 cells, human embryonal kidney 293 cells; HPAEC, high-pH anion-exchange chromatography; PAD, pulsed amperometric detection; rAChE, recombinant acetylcholinesterase; rBoAChE, recombinant bovine acetylcholinesterase; rHuAChE, recombinant human acetylcholinesterase.

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circulatory residence of rHuAChE produced in HEK-293 cells. We use refined analytical approaches to examine the sialylation state of the rHuAChE and demonstrate the limited ability of the cellular glycosylation machinery to generate fully sialylated rHuAChE. We then employ biochemical methods *in vitro* as well as genetic engineering approaches to modulate the sialic acid content and circulatory clearance of AChE by β-galactoside α-2,6-sialyltransferase (α2,6ST; EC 2.4.99.1), and finally we propose an engineered cell system for the production of glycoproteins with extended circulatory residence.

EXPERIMENTAL

Construction of the α2,6ST expression vector

The cDNA coding for α2,6ST was amplified by PCR from rat poly(A)-primed cDNA with the following primers: cggggtacc-ATGATTCATACCAACTTGAAG (upstream primer) and ggcggatcCTCAACAACGAATGTTCCGGA (downstream primer); lower-case nucleotides represent the *Kpn*I and *Bam*HI extensions that were used for ligation. PCR was performed with 30 cycles of the following temperature profile: denaturation for 30 seconds at 94 °C, primer annealing for 30 s at 55 °C and primer extension for 1.5 min at 72 °C. Reactions were set up in a final volume of 100 μl of manufacturer-supplied *Taq* polymerase buffer containing template cDNA, 20 pmol of each primer, 50 μM of each dNTP and 2 units of *Taq* polymerase (Promega). The 1210 bp PCR product was ligated into the corresponding sites of the pCEP4 vector (Invitrogen) downstream of the CMV promoter, to generate pCEP4-α2,6ST. The cloned cDNA was sequenced and its identity was verified by comparison with the published rat liver α2,6ST cDNA sequence [21] (EMBL accession number M18769). DNA sequencing by the dideoxy termination method was performed with Sequenase II (USB).

Transfection and selection of α2,6ST-producing HEK-293 cells

HEK-293 cells, as well as cell clones of the same lineage that expressed high levels of rHuAChE (HEK-293-AChE) were transfected [22] with the pCEP4-α2,6ST plasmid, which also carried a hygromycin resistance marker. Selection of stably transfected cells was performed by incubating the cells in the presence of 0.3 mg/ml hygromycin; colonies of hygromycin-resistant cells that appeared 3 weeks after transfection (approx. 200 colonies per 2×10^6cells per 20 μg of DNA) were pooled and examined for intracellular α2,6ST activity. To isolate individual cell clones expressing α2,6ST, cell pools were cloned by limiting dilution as described previously [18]; 25 individual cell clones were chosen at random, cells were expanded, and clones were tested for α2,6ST activity.

Determination of intracellular sialyltransferase activity

Cell pellets (10^7 cells per sample) were washed twice with PBS, resuspended in 1 ml of lysis buffer containing 0.1 M sodium cacodylate, pH 6, and 1 % (v/v) Triton X-100 and left on ice for 15 min. Cells were sonicated for 30 s on a Microson cell disrupter (Misonix). The cell lysate was centrifuged for 10 min at 1000 ***g*** in an Eppendorf centrifuge. The clear supernatant, containing 10–20 μg/μl protein, was stored in small aliquots at −70 °C until the determination of the sialyltransferase activity. Protein concentration was determined with the bicinchoninic acid kit (Sigma). Sialyltransferase activity was assayed in cellular homogenates by a modification of a method described previously [23]. Up to 200 μg of cell extract was incubated in the presence of 50 μg of acceptor bovine asialofetuin (Sigma) and 1000 pmol of the sialic acid (NeuAc) donor CMP-[^{14}C]NeuAc (approx. 100000 c.p.m. per assay) in a final volume of 50 μl including 0.5 % (v/v) Triton X-100 and 50 mM sodium cacodylate. Incubation was performed at 37 °C for up to 3 h. The assay was terminated and the free radioactive CMP-NeuAc was removed by precipitation with a large excess of ice-cold 10 % (w/v) trichloroacetic acid. Trichloroacetic acid-precipitable material was processed in a liquid-scintillation counter to quantify [^{14}C]NeuAc incorporation. Assays were performed kinetically; the amount of NeuAc incorporated was expressed as pmol of NeuAc transferred/h per mg of protein of cell extract to an excess of asialoprotein acceptor. Standard deviations of less than 10 % were observed. Positive controls were set up with commercial pure rat liver α2,6ST (Boehringer Mannheim).

Source of rAChEs and enzyme purification

rHuAChE or recombinant bovine acetylcholinesterase (rBoAChE) was collected from stable G418® cell clones expressing various levels of wild-type enzyme [7,18] or a rHuAChE derivative carrying at the C-terminus the KDEL-retention signal [24]. Pure rAChEs were prepared by affinity chromatography utilizing procainamide–Sepharose 4B columns as described previously [18]. Eluted enzyme was extensively dialysed against ice-cold PBS and quantified by protein mass determination (bicinchoninic acid kit) and by specific ELISA based on polyclonal antibodies against rHuAChE [22]. Purified rAChEs were used for the determination of sialic acid content, ^{14}C-NeuAc incorporation assays, SDS/PAGE, glycan preparation for high-pH anion-exchange chromatography–pulsed amperometric detection (HPAEC–PAD) and circulatory clearance experiments.

[^{14}C]Sialic acid incorporation into rHuAChE

rHuAChE (20 pmol) was incubated at 37 °C with 500 pmol of CMP-[^{14}C]NeuAc in the presence of 0.2 m-unit of commercial α2,6ST in a buffer containing 0.5 % (v/v) Triton X-100, 50 mM sodium cacodylate and 1 mg/ml BSA in a final volume of 50 μl. Reactions were stopped at various time points (1–8 h) by the addition of 1 ml of ice-cold 10 % (w/v) trichloroacetic acid. Usually, incorporation levelled off after 1–3 h of incubation. Control experiments established that trichloroacetic acid-precipitable radioactivity over the background level was not detected in either the absence of an acceptor protein or in the presence of an acceptor glycoprotein that did not display N-linked glycan terminating in galactose residues (such as asialomucin). Positive control experiments set up with asialofetuin as acceptor showed that, once incorporated, radioactive NeuAc was not chased by an excess of unlabelled CMP-NeuAc donor. The amount of [^{14}C]NeuAc incorporated (expressed as mol of NeuAc/mol of AChE) represents the number of free sites per AChE subunit that were available for the addition of NeuAc.

Sialylation and desialylation of rAChEs *in vitro*

Pure rAChEs (1.8 nmol of human or bovine) were incubated for 20 h at 37 °C in the presence of 2 m-units of α2,6ST (Boehringer) and 100 nmol of CMP-*N*-acetylneuraminic acid/50 mM NaCl (final volume 800 μl). The rHuAChE sialylated *in vitro* was dialysed extensively against water for N-glycan analysis, or against PBS for pharmacokinetics studies *in vivo*. Desialylation of rHuAChE was performed as described previously [4] by agarose-bound neuraminidase (Sigma), which was then removed by Eppendorf centrifugation. Desialylated enzyme was dialysed against PBS to remove free sialic acid.

Glycan structural analysis

Sialic acid content

Determination of sialic acid contents was performed on highly purified enzyme preparations. The release and quantification of sialic acid residues were achieved by the thiobarbituric acid method [25]. Sialic acid extracted into cyclohexanone was quantified at 549 nm alongside a standard curve of *N*-acetylneuraminic acid (Sigma) that had been subjected to the same assay conditions.

Size and charge profiles of the glycans

Size and charge profiles of the glycans associated with rHuAChE produced in human cells of the HEK-293 line were determined by Oxford GlycoSystems (Oxford, U.K.). In brief, the oligosaccharides were released quantitatively by hydrazinolysis from 300 μg of pure protein sample and recovered with a GlycoPrep 1000®. The sample was fluorescently labelled by reductive amination with 2-aminobenzamide followed by purification by paper chromatography. For the determination of charge distribution (see Figure 1B), an aliquot of the pool was subjected to HPLC anion-exchange chromatography on a GlycoSep C column with acetonitrile/ammonium acetate as eluant. To determine the nature of the negatively charged substituents, an aliquot of the total pool of fluorescently labelled oligosaccharides was incubated with *Arthrobacter ureafaciens* neuraminidase and then subjected to GlycoSep C chromatography. For determination of the size distribution, the total pool of deacidified 2-aminobenzamide-labelled oligosaccharides was subjected to high-resolution gel-permeation chromatography with the GlycoSequencer. The material was eluted with water; on-line detection was achieved by a fluorescence flow detector (for the 2-aminobenzamide-labelled oligosaccharides) or by a differential refractometer (for individual glucose oligomer markers). The hydrodynamic volume of the released glycans is expressed in glucose units; this was used as an estimate of the molecular mass of the glycans (e.g. 12 glucose units is equivalent to a molecular mass of 2000 ± 20 Da).

HPAEC–PAD analysis of N-glycans

Highly purified rHuAChE preparations (100 μg) were extensively dialysed against water and freeze-dried. Hydrazine-based deglycosylation of N-linked glycans and further purification steps were performed by the automated Glycoprep100 instrument (Oxford GlycoSciences, Oxford, U.K.). The purified glycans, dissolved in 4.5 ml of 1 M acetic acid, were dried by a rotary vacuum evaporator. The dried glycan pool was resuspended in 100 μl of 4 μg/μl isomaltotriose (Sigma) in water; a 90 μl portion was loaded on a PA-100 column (Dionex Corp., Sunnyvale, CA, U.S.A.) for separation by using the DX-500 HPAEC–PAD system (Dionex Corp.). Glycans were eluted with a gradient of sodium acetate in 100 mM NaOH, and detected by a pulsed amperometric detector. Assignment of peaks in the resultant chromatogram was achieved with standard oligosaccharide markers (Cat. No. A1, A1F, A2, A2F, NA2F, A3; Oxford GlycoSystems). Isomaltotriose served as an internal marker to ensure the consistency of the various runs.

Enzyme activity

AChE activity was measured by the method of Ellman et al. [26]. Assays were performed in the presence of 0.5 mM acetylthiocholine, 50 mM sodium phosphate buffer, pH 8.8, 0.1 mg/ml BSA and 0.3 mM 5,5′-dithiobis-(2-nitrobenzoic acid). The assay was performed at 27 °C and monitored with a Thermomax microplate reader (Molecular Devices). K_m values for acetylthiocholine were obtained from Lineweaver–Burk plots and k_{cat} calculations were based on polyclonal ELISA measurements [22]. Inhibition constants (K_i) for the specific inhibitors edrophonium, propidium, decamethonium and BW284C51 were derived as described previously [19].

Pharmacokinetics

Clearance experiments *in vivo* in mice (three to six ICR male mice per enzyme sample) and analyses of pharmacokinetics profiles were performed as described by Kronman et al. [4]. The study was approved by the local ethical committee on animal experiments. Residual AChE activity in blood samples was measured and all values were corrected for background activity determined in blood samples withdrawn 1 h before the experiment was performed. The clearance patterns of the various enzyme preparations were biphasic and fitted to a bi-exponential elimination pharmacokinetic model as described previously [4].

RESULTS

Analysis of N-glycans of rHuAChE produced by HEK-293 cells

As an initial step towards understanding the possible contribution of N-glycans to circulatory residence of rHuAChE, the carbohydrate side chains associated with rHuAChE produced in HEK-293 cells were subjected to a structural analysis comprising the determination of size, charge, antennary branching and sialylation extent. Chromatographic size determination (Figure 1A) established that the main fraction (more than 80 %) of the deacidified glycans released from a pure preparation of the HEK-293 cell-generated rHuAChE had a size equivalent to 12.2 ± 0.1 glucose units (molecular mass 2000 ± 20 Da), in agreement with that of a bisected biantennary glycan projection containing fucose (see also Figure 2). The results also indicate that rHuAChE produced in this cell system did not carry significant amounts of O-glycans, as suggested by the absence of any substantial peak within the small-size (1–5 glucose units) region of the chromatogram (Figure 1A). Charge analysis of the glycan pool released from rHuAChE (Figure 1B, left panel) before deacidification by treatment with neuraminidase revealed that the rHuAChE-associated glycans consisted of a mixed population of both charged and uncharged species. After treatment of the oligosaccharide preparation with bacterial neuraminidase, more than 99 % of the material appeared in the uncharged peak (Figure 1B, right panel), demonstrating that nearly all of the charge was contributed by terminal sialic acid residues. To allow high-resolution analysis of the N-glycans, rHuAChE was subjected to hydrazine-based deglycosylation and the released glycans were separated by HPAEC on a PA-100 column. The analyses (Figure 2) revealed the presence of four different groups of N-glycans: (a) asialylated biantennary, (b) monosialylated biantennary, (c) disialylated biantennary and (d) and trisialylated triantennary (Figure 2). Integration of peak areas of the eluted oligosaccharides confirmed that most of the glycans (more than 85 %) corresponded to biantennary forms, whereas only a minor fraction was triantennary.

Taken together, the carbohydrate analyses demonstrate that: (1) N-glycans associated with rHuAChE produced in HEK-293 cells are relatively homogeneous both in size and in branching, in contrast with the heterogeneous nature of the N-glycans observed in other recombinant cholinesterases [6]; (2) N-glycans associated

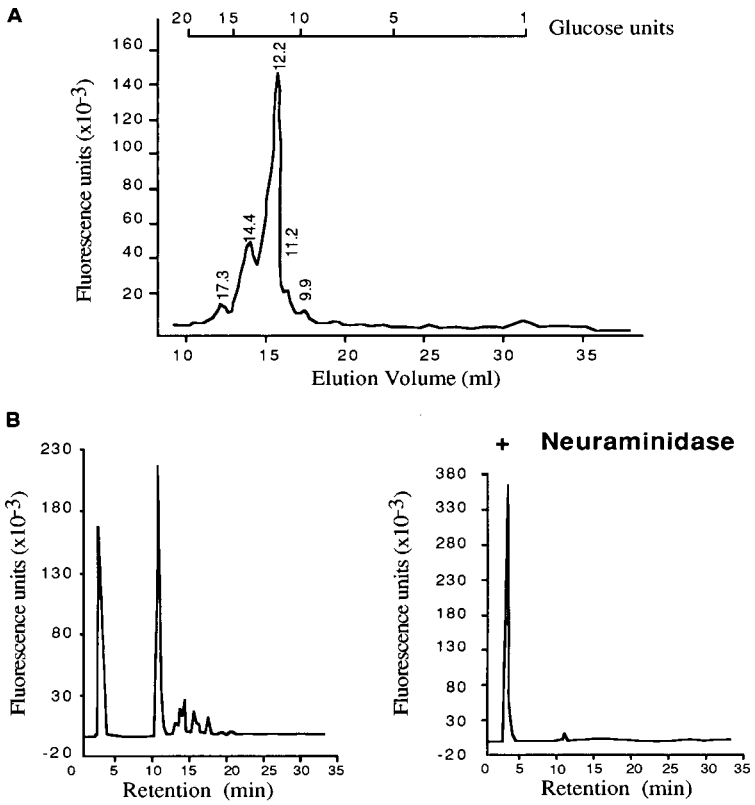

Figure 1 Structural analysis of carbohydrates released from rHuAChE

(**A**) The size profile of the total pool of neuraminidase-digested oligosaccharides was determined by high-resolution gel-permeation chromatography on a Glyco-sequencer. The hydrodynamic volume of individual 2-aminobenzamide-labelled oligosaccharides is measured in glucose units (see the Experimental section). Numerical superscripts represent the elution position of the co-applied glucose oligomers, expressed in glucose units and corrected for the contribution of the fluorescent label 2-aminobenzamide to the position of the elution peak. (**B**) Charge distribution of the total pool of oligosaccharides, before (left panel) and after (right panel) neuraminidase treatment, determined by HPLC GlycoSep C anion-exchange chromatography.

with HEK-293 cell-produced rHuAChE are mostly of the biantennary type; and (3) the N-glycans are only partly sialylated, approx. 60% of them carrying terminal sialic acid residues. These results are in good agreement with the sialic acid measurements reported previously [4], suggesting that most glycans associated with rHuAChE produced by HEK-293 cells are partly sialylated.

rHuAChE can serve as a substrate for α2,6ST

In view of the incomplete sialylation of rHuAChE generated in HEK-293 cells, we addressed the question of whether rHuAChE can act as a sialic acid acceptor in a α2,6ST reaction. This could be useful both as an analytical assay that scores the number of sites available for sialylation as well as a way of increasing the sialic acid content of the recombinant enzyme.

Incubation of purified rHuAChE with the radiolabelled NeuAc donor (CMP-NeuAc) in the presence of α2,6ST (also named ST6Gall [27]) resulted in the addition of NeuAc residues to rHuAChE (Figure 3A). Maximal sialylation was reached after approx. 2 h, at an incorporation of 1.8 ± 0.3 mol of NeuAc/mol of acceptor rHuAChE (Figure 3A). Because direct sialic acid measurement indicated that HEK-293-generated rHuAChE contained 3.4 ± 0.5 mol per enzyme subunit (Figure 3B, right panel), the total amount of sialic acid after incorporation *in vitro* (5.2 ± 0.5 mol per enzyme subunit) was close to that expected for full sialic acid occupancy of the three biantennary N-glycans associated with rHuAChE.

Sialylation of rHuAChE in HEK-293 cells is influenced by its level of expression

The partial sialylation of rHuAChE produced in HEK-293 cells might be a result of either endogenous sialidase activity that removes some of the sialic acid moieties [28–30] or of inefficient sialylation of the recombinant product.

To distinguish between these two possibilities, we measured the level of sialylation in rHuAChE produced by two HEK-293 cell lines that exhibited a 20-fold difference in levels of expression of the recombinant enzyme. Preparations of pure rHuAChE generated by 'high' (50 units/24 h per ml) and 'low' (3 units/24 h per ml) rHuAChE producer HEK-293 cell clones were assayed for incorporation of [^{14}C]NeuAc. It was found that rHuAChE from the 'high' producer clone allowed the incorporation of 1.8 ± 0.3 NeuAc residues per AChE subunit; rHuAChE from the 'low' producer clone exhibited the incorporation of only 0.5 ± 0.1 NeuAc residues per subunit (Figure 4B). SDS/PAGE analysis of pure rHuAChE generated by 'high' and 'low' producer HEK-293 cell clones revealed that both enzymes possessed similar electrophoretic migration profiles before and after treatment with N-glycanase (Figure 4A), suggesting that the overall glycosylation pattern was not affected by the rate of expression of the recombinant polypeptide. Nevertheless it should be noted

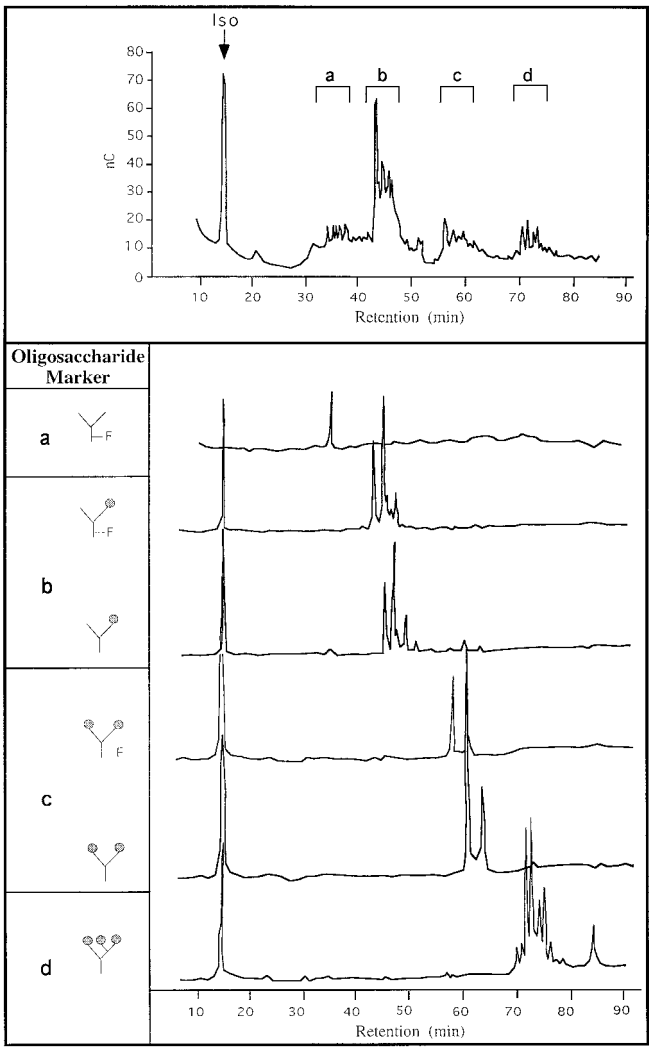

Figure 2 HPAEC–PAD analysis of carbohydrates released from rHuAChE

Upper panel: N-glycan profile of rHuAChE. Purified N-glycans from 100 μg of rHuAChE were fractionated on a PA-100 ion-exchange column connected to a Dionex 500 system. The elution time regions of (a) asialoglycan, (b) monosialylated biantennary, (c) disialylated biantennary and (d) trisialylated triantennary oligosaccharide markers (see below) are indicated. The position of the isomaltotriose internal marker is also indicated. Lower panels: elution profiles of defined oligosaccharide standards. The elution positions of individual complex-type oligosaccharide markers with or without fucose substitutions were determined. The structures of these glycan markers are shown schematically at the left. Fucose core substitution is represented by a short projection marked F and sialic acid terminal residues are represented by a grey circle. Under the conditions used, all asialoglycans were eluted within a narrow range (32–37 min). This group is represented by the biantennary-fucosylated asialoform.

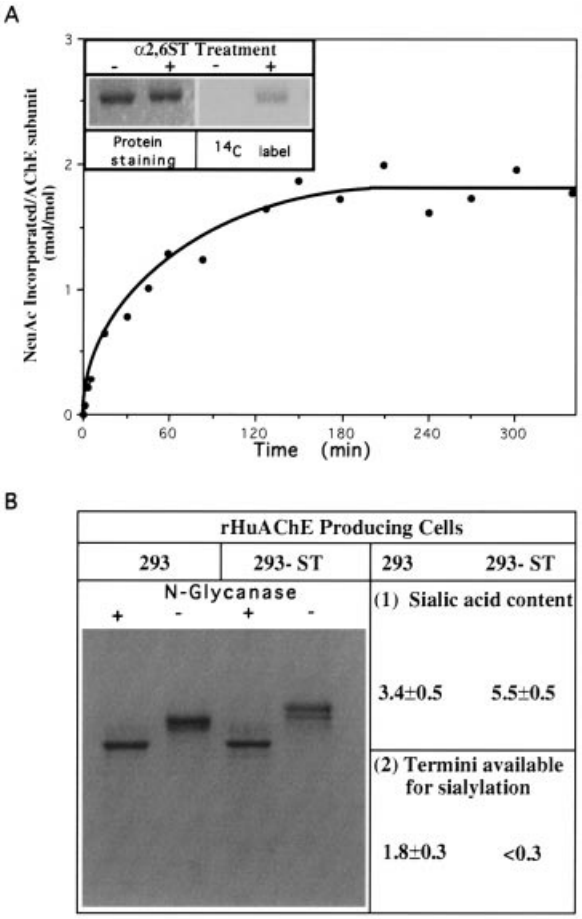

Figure 3 Incorporation of sialic acid residues into rHuAChE *in vitro* and *in vivo*

(**A**) Treatment of rHuAChE *in vitro* with α2,6ST: kinetics of incorporation of ^{14}C-labelled CMP-NeuAc into purified rHuAChE. The c.p.m. were corrected for background trichloroacetic acid-precipitable radioactivity observed at zero time. Inset: SDS/PAGE before and after 3 h of incubation in the presence of 2,6-ST. Coomassie Blue staining (left) and autoradiograph (right) of each gel are shown. (**B**) Sialic acid occupancy of rHuAChE produced in HEK-293 cells and HEK-293 cells co-expressing the rat α2,6ST gene. Pure preparations of rHuAChE produced by HEK-293 cells or α2,6ST-engineered HEK-293 cells, before and after N-glycanase treatment, were analysed by SDS/PAGE (left panel). The state of sialylation of the two preparations of rHuAChE was evaluated by: (1) direct determination of sialic acid content by the thiobarbituric acid method and (2) determination of termini available for sialylation [by the method described for panel (**A**)].

that rHuAChE from the low producer (before N-glycanase treatment) exhibited a slightly lower mobility, consistent with its increased level of sialic acid content.

Because terminal sialylation affects the circulatory retention of rHuAChE [4], we used this indirect but sensitive pharmacokinetic assay to determine whether the dissimilarities in sialic acid content of rHuAChE from the 'low' and 'high' producer clones was reflected in a coinciding differential circulatory clearance. Figure 4(C) shows that rHuAChE synthesized by the 'low producer' clone was retained longer in the circulation than rHuAChE generated by the 'high producer' clone. Notably, the circulatory half-life of the 'low' producer enzyme, in the second (slow) phase of the clearance curve, described by the $t_{\frac{1}{2}}\beta$ value, was significantly higher (133 ± 13 min) than that of the 'high' producer enzyme (80 ± 4 min) (Table 1). This result is in accordance with the ^{14}C incorporation studies, which established that in the 'low' producer rHuAChE more glycan termini were occupied by sialic acid residues (Figure 4B). If cellular sialidase activity were responsible for sialic acid trimming, then rHuAChE generated by a low-level expressor clone would have displayed fewer terminally sialylated glycans than rHuAChE generated by a high-level expressor clone and consequently also a shorter residence time. These results therefore strongly favour the idea that partial sialylation of HEK-293-generated rHuAChE observed in 'high' producer clones is due to inefficient sialylation rather than sialidase activity.

Terminal glycan sialylation, which occurs in the *trans*-Golgi apparatus, is influenced by the residence time of the substrate

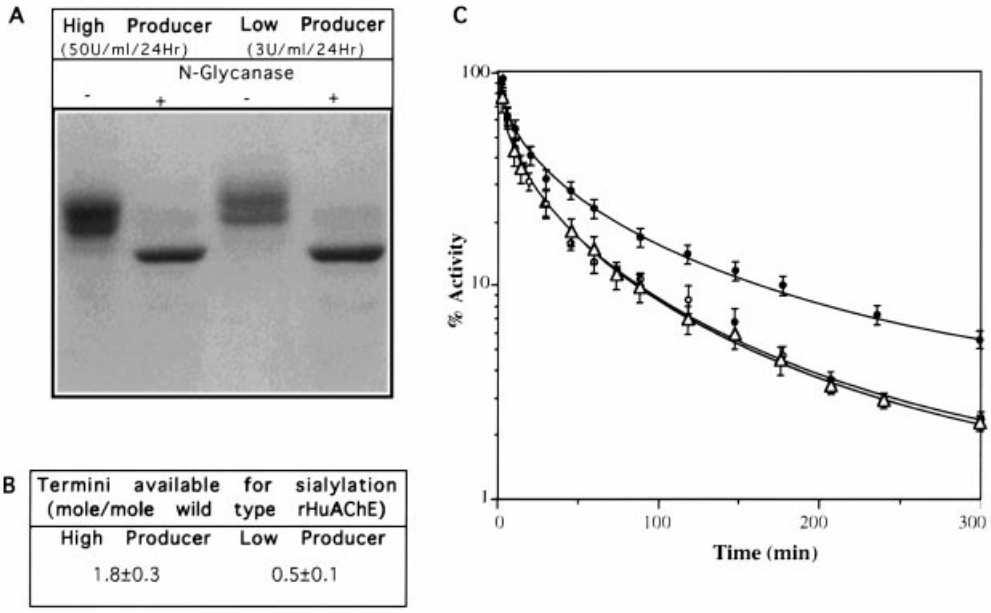

Figure 4 Comparison of enzymes secreted from 'low' and 'high' producer HEK-293 cell clones

Recombinant enzyme was purified from stably transfected HEK-293 clones expressing high levels of wild-type rHuAChE (50 units/24 h per ml), high levels of rHuAChE-KDEL (60 units/24 h per ml), and low levels of wild-type rHuAChE (3 units/24 h per ml). (**A**) SDS/PAGE of wild-type rHuAChE untreated or treated with N-glycanase. (**B**) Evaluation of the sialic acid occupancy of rHuAChE from the two wild-type clones (see the Experimental section). (**C**) Circulation clearance profile of wild-type rHuAChE generated by a 'low' producer clone (●), wild-type rHuAChE generated by a 'high' producer clone (○) and rHuAChE-KDEL produced by 'high' producer clone (△). Values are averages of residual AChE activities determined in three independent experiments.

Table 1 Pharmacokinetic parameters of rHuAChEs expressed in HEK-293 cells differing in their sialic acid contents

Production levels of rHuAChE were defined as high (50 units/24 h per ml) and low (3 units/24 h per ml). Sialylation modulation *in vitro* was performed with rHuAChE purified from a high producer cell clone incubated in the presence of commercial α2,6ST. Sialylation modulation *in vivo* refers to enzyme isolated and purified from HEK-293 cell line cultures producing high levels of rHuAChE and stably transfected with the rat α2,6ST. Clearance profiles conform to the biphasic elimination curve in the form $C(t) = A\exp(-k_\alpha t) + B\exp(-k_\beta t)$ Pharmacokinetic data relate to the B (slow) phase. In the fast phase (A), all k values were within $(8–9) \times 10^{-2}$ min^{-1}. The fraction of enzyme cleared during the fast phase (A) was within the range 63–74%, as reflected by the complementary B values ($A + B = 100$). $t_{\frac{1}{2}} = \ln 2/k\beta$. Results are means ± S.E.M.

Production level of rHuAChE	Sialylation modulation		Pharmacokinetic parameters	
	In vitro	*In vivo*	B (%)	$t_{\frac{1}{2}}$ (min)
High	−	−	25 ± 1.5	80 ± 4
Low	−	−	37 ± 5	133 ± 13
High	+	−	37 ± 2	138 ± 14
High	−	+	42 ± 2	129 ± 9

glycoprotein in this compartment and by the intracellular sialyltransferase concentration [31]. In previous studies we have shown that rHuAChE expressed in HEK-293 cells traverses the Golgi compartments rapidly [32], yet the addition of the KDEL endoplasmic reticulum retention sequence to the C-terminus of rHuAChE results in its sequestration in the Golgi [24]. However, the KDEL-modified rHuAChE, generated in a 'high' producer clone (60 units/24 h per ml) exhibited profiles indistinguishable from those of wild-type rHuAChE in either SDS/PAGE (results not shown) or circulatory clearance (Figure 4C). Thus the undersialylated state of the rHuAChE N-glycans was not redressed by simply altering its Golgi residence time by the addition of KDEL. Taken together, the results indicate that undersialylation of rHuAChE produced by high-level expressor clones is a consequence of a quantitative limitation of endogenous sialyltransferases. Indeed, examination of sialyltransferase activity levels in HEK-293 cells (Figure 5, right panel) indicated that these are no more than 5% of those found in cells of hepatic origin [9,10], which are known to promote the efficient sialylation of glycoproteins.

Engineering cells expressing elevated levels of α2,6ST

Because HEK-293 cells are limited in their sialylation ability, and rHuAChE produced by these cells can serve as a target for the addition of sialic acid residues via α2,6ST activity (Figure 3), we decided to increase the level of rHuAChE N-glycan sialylation by expressing a heterologous gene for α2,6ST into cells producing high levels of rHuAChE.

A cDNA coding for α2,6ST was cloned by PCR amplification from rat liver cDNA. The α2,6ST sequence, spanning the entire coding segment, was confirmed by comparison with the published rat α2,6ST sequence [21]. Sequencing revealed that the cloned gene represented the liver-specific version of α2,6ST, by virtue of the characteristic cysteine residue at position 123 (Figure 5, left panel). This α2,6ST form is retained in the Golgi compartment [33] and thus possesses an increased ability to act on cellular glycoproteins. The alternative α2,6ST form, which harbours a tyrosine residue at that position, should be secreted into the medium [33] and therefore would not have been suitable for our purpose.

The α2,6ST cDNA was cloned into an expression vector carrying the hygromycin resistance gene and used to transfect HEK-293 cells producing and secreting high levels of rHuAChE.

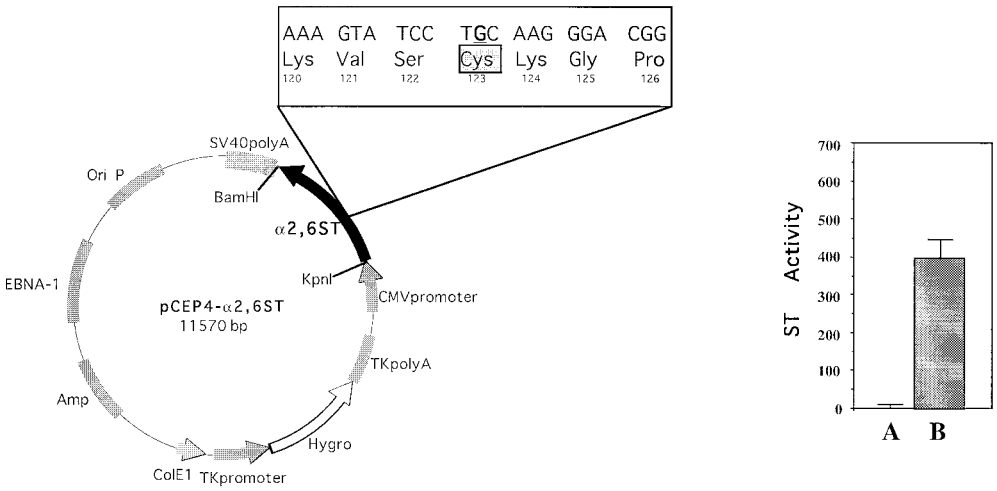

Figure 5 α2,6ST expression system

Left panel: plasmid pCEP4-α2,6ST carrying the cDNA of the 'Cys' version [33] of rat α2,6ST. Right panel: sialyltransferase activity (expressed as pmol of NeuAc transferred/h per mg of protein) in naive (column A) or α2,6ST-transfected (column B) HEK-293-derived cell lines. Results are average activities obtained in triplicate experimental groups and are normalized to the total protein content of the cellular extract.

Selection for cells stably expressing the gene for α2,6ST was based on hygromycin resistance because rHuAChE-producing cells harbour an integrated copy of the neomycin resistance gene, which was utilized for selecting rHuAChE expressor cells [18]. Stably transfected cells were subjected to limiting-dilution cloning to permit the isolation of pure cell clones expressing the gene for α2,6ST.

Cell extracts of an individual α2,6ST expressor cell clone, 293-AChE-ST, were prepared and tested for sialyltransferase activity. A significant increase in sialyltransferase activity over the background level (at least 20-fold higher) was observed in the 293-AChE-ST cells (Figure 5, right panel). In line with the known substrate specificity of the α2,6ST enzyme [34,35], asialofetuin (which contains Gal-β1-4 GlcNAc groups on N-linked oligosaccharides) but not asialomucin (which contains GalNAc groups found as O-linked oligosaccharides) could serve as a NeuAc acceptor in the assay.

To determine whether or not the α2,6ST activity in the transfected cells was compromised by the concomitant expression of rHuAChE, we compared the α2,6ST levels of two cell lines: HEK-293 cells transfected only with the gene for α2,6ST and the previously described cells (293-AChE-ST), which co-expressed the genes for both α2,6ST and AChE. Sialyltransferase activity levels in these cell clones were found to be similar, confirming that AChE production had no effect on the levels of production of α2,6ST (Figure 5, right panel). Furthermore rHuAChE levels in cells co-expressing the gene for α2,6ST together with the gene for AChE were similar to those in cells transfected with the gene for AChE only, showing that the ability to produce AChE was not affected by introduction of the gene for α2,6ST and/or by the acquisition of hygromycin resistance.

Biochemical characteristics of rHuAChE produced by α2,6ST-engineered cells

The activity and reactivity of rAChE towards various ligands were not affected by introduction of the gene for α2,6ST. K_m and k_{cat} values for acetylthiocholine were 0.12 mM and 3.9×10^5/min respectively. Inhibition constants (K_i) for the specific inhibitors edrophonium, propidium, decamethonium and BW284C51 were 0.4, 0.2, 2 and 2.5 μM respectively. These values are similar to those determined for the rHuAChE produced by HEK-293 cells that do not express the heterologous gene for α2,6ST [36]. When subjected to SDS/PAGE analysis, the 293-AChE-ST product displayed a slower electrophoretic mobility than that of rHuAChE produced in unmodified HEK-293 cells (Figure 3B, left panel), which is consistent with an increased content of sialic acid. Except for the apparent differences in mobility, the enzyme products of 293-AChE-ST cells and unmodified HEK-293 cells exhibited a similar electrophoretic profile, consisting of the two characteristic bands [37] representing rHuAChE glycoforms that are sensitive to treatment with N-glycanase. This suggests that the overall glycosylation pattern of rHuAChE was not affected by co-expression of the gene for α2,6ST.

The sialic acid content of rHuAChE purified from 293-AChE-ST cells was found to correspond to a NeuAc-to-AChE molar ratio of 5.5 ± 0.5 (Figure 3B, right panel). In the same analyses, rHuAChE purified from HEK-293 cells not transfected with α2,6ST exhibited a sialic acid content equivalent to a NeuAc-to-AChE molar ratio of 3.4 ± 0.5. Thus AChE produced by cells co-expressing a heterologous gene for α2,6ST were sialylated to a greater extent than that produced by unmodified HEK-293 cells, reflecting the acquisition of a higher sialylation potential by the α2,6ST-transfected cells. Notably, the increase in sialic acid content of the α2,6ST-engineered cell product (2.1 mol of NeuAc/mol of AChE) was in good agreement with the number of sialic acid residues that could be added *in vitro* to rHuAChE produced by HEK-293 cells not transfected with the gene for α2,6ST. Moreover, when rHuAChE produced by the 293-AChE-ST cells was subjected to an *in vitro* incorporation assay (Figure 3B, right panel), only marginal levels of NeuAc were accepted (less than 0.3 mol of NeuAc/mol of AChE, compared with 1.8 ± 0.3 mol of NeuAc/mol of AChE incorporated into rHuAChE produced in HEK-293 cells). These results suggest that the α2,6ST engineered cells promoted efficient sialylation of rHuAChE *in vivo*.

The glycans associated with rHuAChE produced by 293-AChE-ST cells were further subjected to direct oligosaccharide

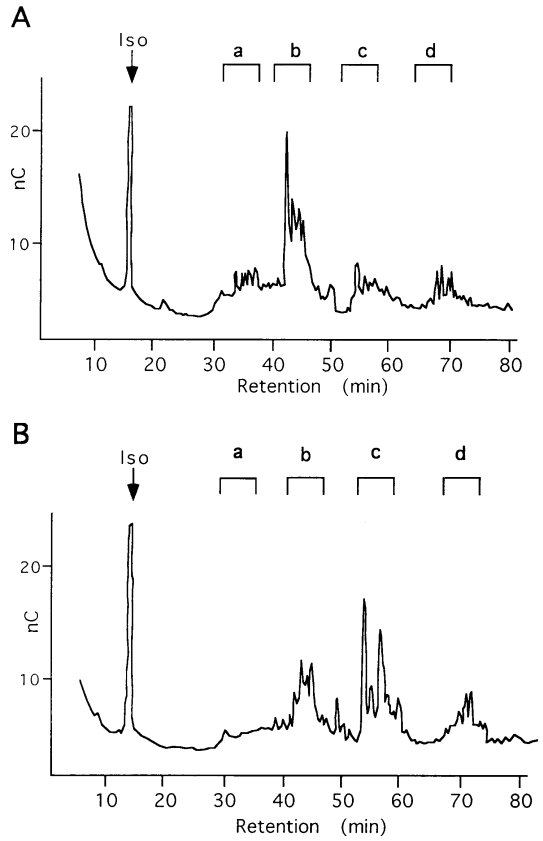

Figure 6 Comparison of HPAEC–PAD profiles of carbohydrates released from rHuAChE produced by HEK-293 cells and α2,6ST-engineered HEK-293 cells

HPAEC–PAD carbohydrate profiles of rHuAChE produced by HEK-293 cells (**A**) and by α2,6ST-engineered HEK-293 cells (**B**). The letters a, b, c and d denote the elution time regions of asialylated, monosialylated, disialylated and trisialylated glycans respectively, as described in the legend to Figure 2. The peak denoted Iso arose from the internal marker isomaltotriose, which was added to every sample. See Table 2 for the relative percentage area of each glycan in the chromatogram.

profiling by HPAEC–PAD and compared with the profile of rHuAChE produced in unmodified HEK-293 cells (Figure 6 and Table 2). As was noted for rHuAChE produced in HEK-293 cells not transfected with α2,6ST, the glycans associated with rHuAChE produced by 293-AChE-ST cells were predominantly of the biantennary type. However, the distribution of the various N-glycan sialoforms in the two rHuAChE preparations was markedly altered. Whereas the biantennary glycans of rHuAChE from non-α2,6ST-transfected HEK-293 cells exhibited a percentage area distribution of asialylated, monosialylated and disialylated forms of approx. 15:60:16, the respective distribution in rHuAChE produced by 293-AChE-ST cells was 5:39:43 (Table 2). Thus the rHuAChE produced by 293-AChE-ST cells exhibited a significantly lower level of asialylated and monosialylated biantennary structures and a concomitant higher level of fully sialylated biantennary N-glycan forms. In fact the relative percentage areas of the fully sialylated biantennary form in cells co-expressing the gene for α2,6ST were approx. 3-fold those in unmodified cells. In addition, a small increase in trisialylated forms was also displayed in rHuAChE of 293-AChE-ST cells (14% of total area, as opposed to 9% in rHuAChE of unmodified HEK-293 cells).

Most notably, a similar alteration in the glycan chromatographic profile was observed when the rHuAChE product from unmodified HEK-293 cells was subjected to *in-vitro* sialylation by incubation with pure α2,6ST. After such a treatment, the percentage area distribution of asialylated, monosialylated and disialylated forms of the biantennary N-glycans was approx. 4:34:47 [compare Figure 7(A) with Figure 6(A); see also Table 2]. These results clearly indicate that the genetic modulation of rHuAChE producer cells by the introduction of a heterologous gene for α2,6ST promoted highly efficient sialylation. Moreover, when the rHuAChE product of 293-AChE-ST cells was also subjected to sialylation *in vitro* (Figure 7B), the glycan pattern remained virtually unchanged [compare Figure 7(B) with Figure 6(B); see also Table 2], suggesting that sialylation *in vivo* led to almost full occupation of glycan termini that were available for sialic acid addition. However, a certain fraction of the biantennary N-glycans remained in the monosialylated form, suggesting that some of the N-glycan termini were refractive to sialylation. This fraction, representing approx. 20% of N-glycan termini, was observed both in the enzyme oversialylated *in vitro* and in the enzyme produced *in vivo* by α2,6ST-engineered cells, suggesting that the inability to undergo additional sialylation was due to some structural aspect of the corresponding N-glycan termini (see the Discussion section).

rAChEs produced by α2,6ST-engineered cells or subjected to α2,6ST sialylation *in vitro* display an enhanced circulatory residence

Purified enzyme isolated from 293-AChE-ST cells was administered to mice, and the pharmacokinetics profile was determined

Table 2 Quantitative analysis of HPAEC percentage area of various N-glycan forms of rHuAChE

The normalized integrated peak areas from Figure 6 (columns 1 and 2) and Figure 7 (columns 3 and 4) are expressed as percentages (100% = total area under the curve). Note the similarity of the values in columns 2, 3 and 4.

		Area of peak (% of total)			
Glycan form	Source of rHuAChE ...	HEK-293	HEK-293-ST	HEK-293 + *in vitro* α2,6ST	HEK-293-ST + *in vitro* α2,6ST
(a) Asialylated		15.4	4.7	4.2	5
(b) Monosialylated		59.6	38.7	34.2	33.7
(c) Disialylated		15.8	42.6	47.5	46.3
(d) Trisialylated		9.2	14	14	15

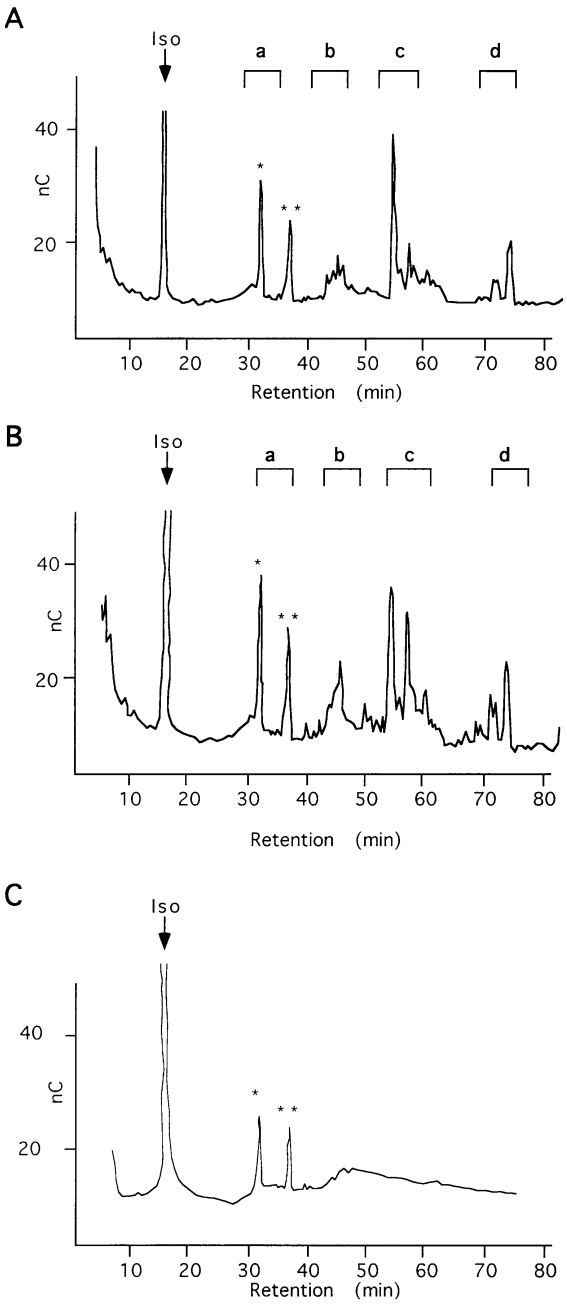

Figure 7 Comparison of HPAEC–PAD profiles of carbohydrates released from different rHuAChE preparations following treatment with α2,6ST *in vitro*

Purified rHuAChE (100 μg) produced by HEK-293 cells (**A**) and by α2,6ST-engineered HEK-293 cells (**B**) were subjected to treatment with commercial rat liver α2,6ST in the presence of excess CMP-NeuAc. The letters a, b, c and d denote the elution time regions of asialylated, monosialylated, disialylated and trisialylated glycans respectively, as described in the legend to Figure 2. Integration of the various peaks was used for calculating the relative percentage area of each glycan in Table 2. The peaks denoted by one and two asterisks represent residual free NeuAc and CMP-NeuAc (NeuAc donor) respectively, which originate from the α2,6ST reaction mix *in vitro*, as established by HPAEC–PAD of appropriate standards shown in (**C**). These peaks do not represent rHuAChE-associated glycans and were not included in the calculations of peak areas in Table 2.

(Figure 8A and Table 1). A significant enhancement of the circulatory retention, reflected by $t_{\frac{1}{2}}\beta$, which describes the slow-phase half-life of the enzyme in circulation (129 ± 9 min for the 293-AChE-ST enzyme, compared with 80 ± 4 min for the under-sialylated enzyme) was observed. When subjected to sialidase treatment *in vitro*, the 293-AChE-ST enzyme was cleared rapidly from the circulation in a manner similar to that of sialidase-treated enzyme of the normal HEK-293 cells (Figure 8A), proving that the enhanced ability of the enzyme oversialylated *in vivo* to be retained in the circulation stemmed from its improved NeuAc content.

The pharmacokinetic profile of rHuAChE produced by the α2,6ST-engineered cells was found to be virtually identical with that of rHuAChE from unmodified HEK-293 cells sialylated *in vitro* (Figure 8B and Table 1), suggesting that the genetic manipulation of the HEK-293 cells' glycosylation machinery by the introduction of a heterologous glycosyltransferase was highly efficient.

The effect of manipulation *in vitro* on AChE clearance can also be demonstrated in rBoAChE. Recombinant bovine AChE produced in HEK-293 cells is cleared rapidly from the circulation, faster than rHuAChE ($t_{\frac{1}{2}}\beta$ for rBoAChE is 29 min) [7]. When subjected to treatment with α2,6ST *in vitro*, rBoAChE displayed an extended circulatory residence (Figure 8C). Notably, the circulatory retention profiles of both the oversialylated bovine and human rAChE forms were nearly identical ($t_{\frac{1}{2}}\beta$ approx. 130 min; Figures 8B and 8C). The fact that both proteins display identical pharmacokinetic values after oversialylation suggests that cell-dependent N-glycan processing might impose an upper limit on the manipulation of circulatory residence by sialylation (see the Discussion section).

DISCUSSION

Structure of glycans of rHuAChE expressed in HEK-293 cells

Cholinesterases differ in their numbers of potential N-glycosylation sites: human and murine AChE carry three such sites [1] and bovine serum AChE carries four sites for N-glycosylation [7], whereas human butyrylcholinesterase carries nine N-glycosylation consensus sequences [38]. Recently, studies addressing the role of N-glycans and other factors in determining the circulatory residence of cholinesterases from different sources provided pertinent information on the structural characteristics of cholinesterase-associated glycans [4–6]. Cholinesterases from various sources [6] were found to differ in a large number of variables, such as subunit oligomerization, N-glycan branching and oligosaccharide sialylation; it was therefore difficult to evaluate conclusively the contribution of N-glycan content and structure to clearance.

In the present study we demonstrate that the major proportion of the N-glycans associated with rHuAChE expressed in HEK-293 cells are of a uniform size (Figure 1A), belonging to the biantennary type as confirmed by gel-permeation chromatography and HPAEC–PAD (Figures 1 and 2 and Table 2). This finding is in accordance with the known propensity of HEK-293 cells to generate glycoproteins bearing complex-type biantennary glycans [39,40]. We have noted previously that only a very minor proportion of the rHuAChE molecules contain O-glycans [4]. This is also reflected, in this study, by the gel-permeation chromatogram of rHuAChE-associated glycans (Figure 1A), which fails to detect any significant peaks characteristic of O-glycans in the small-size range (less than five glucose units [41]). The structural analysis reported in the present study establishes that, although most of the N-glycan forms (more than 85%) exhibit negatively charged sialic acid, the major proportion of these glycans contain only one sialic acid residue per glycan unit, indicating an overall undersialylation of the N-glycans (Figure 2

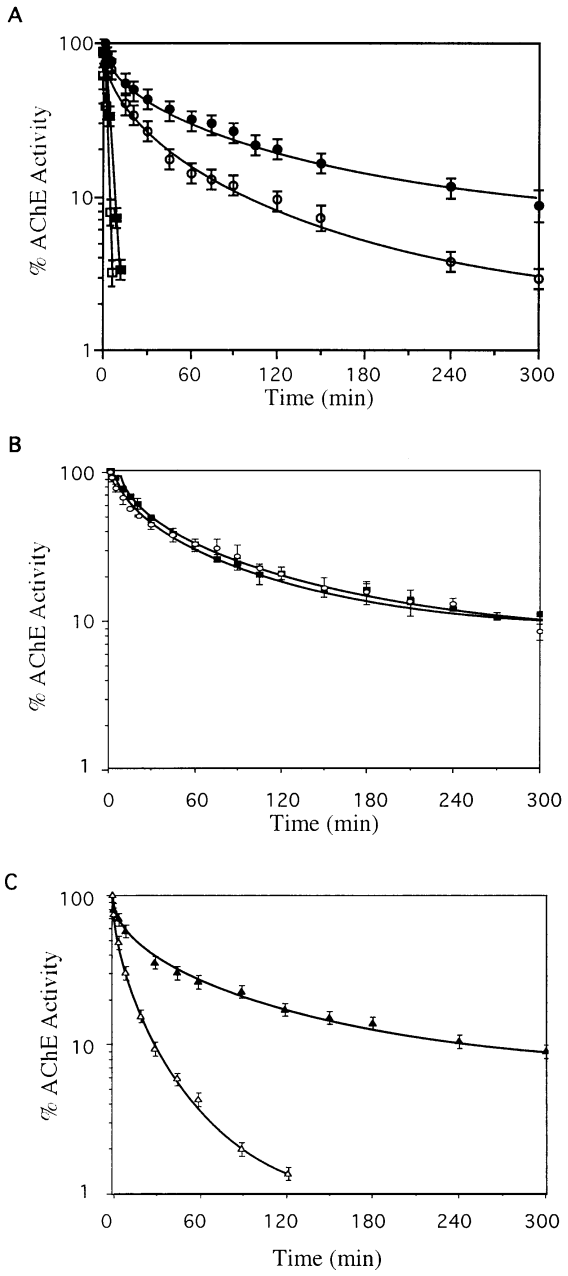

Figure 8 Comparison of circulatory clearance rates of partly sialylated and oversialylated forms of rAChEs

(**A**) Clearance profile of rHuAChE generated by 2,6ST-engineered HEK-293 cells [before (●) or after (■) sialidase treatment] compared with rHuAChE generated by unmodified HEK-293 cells [before (○) or after (□) sialidase treatment]. (**B**) Clearance profile of rHuAChE generated by α2,6ST-engineered HEK-293 cells (○) compared with rHuAChE generated by HEK-293 cells and subsequently oversialylated by treatment *in vitro* with commercial α2,6ST (■). (**C**) Clearance profile of rBoAChE generated by HEK-293 cells before (△) or after (▲) α2,6ST treatment *in vitro*.

and Table 2). This limitation in sialic acid content can be relieved by adding sialic acid residues through the activity of α2,6ST, which leads to a significant transition of the major glycan fraction from the monosialylated to the disialylated form, as attested by HPAEC–PAD analysis (Figures 6 and 7).

The relatively high degree of homogeneity noted for the N-glycans released from rHuAChE produced by HEK-293 cells (Figure 1) is in contrast with the rather heterogeneous nature observed for the N-glycans associated with murine rAChE produced in the same cell line [6]. This difference might be a consequence of amino acid differences, as well as variations in cell growth conditions, enzyme-harvesting regime and storage conditions of the pure protein, all of which are known to affect the quality of the glycans.

Relationship between the extent of rHuAChE glycosylation and sialylation and its circulatory lifetime

Clearance of proteins from the circulation might involve one or multiple removal systems that interact either with epitopes within the protein itself or with elements generated by post-translational modifications such as oligosaccharide appendages [8,42–45]. Cholinesterases are attributed a therapeutic potential by virtue of their bioscavenging ability. Effective exploitation of this potential requires that exogenously administered enzyme be retained in the circulation for a considerable duration. However, rHuAChE is removed from the circulation more rapidly than native serum-derived cholinesterases such as fetal bovine serum AChE and human serum butyrylcholinesterase [4].

To identify the parameters determining the fate of AChE in the circulation, one should rely on a system in which the various AChEs are derived from the same source and each of the potential variables on the molecule can be modified separately and sequentially (e.g. N-glycosylation, oligomerization, charge). We have developed such a system on the basis of a comparison of various mutated forms of rAChEs, all expressed in HEK-293 cells. We have shown [4] that a mutated version of rHuAChE containing only one N-glycosylation site, as opposed to three N-glycosylation sites in the wild-type enzyme, was cleared rapidly from the circulation, suggesting the involvement of N-glycans in circulatory residence. However, an examination of over-glycosylated rHuAChE mutants, containing four or five utilized N-glycosylation sites, revealed that the number of N-glycan appendages does not in itself contribute to circulatory longevity. The evaluation of an array of rHuAChE mutants differing in their sialic acid content allowed us to establish that an inverse linear correlation exists between the number of non-sialylated N-glycan termini and circulatory residence of rHuAChE expressed in HEK-293 cells [4]. In the present study we extend these observations and show that the addition of sialic acid residues to N-glycan termini of rHuAChE by co-expression of recombinant α2,6ST in HEK-293 cells does indeed result in an increased circulatory residence ($t_{\frac{1}{2}}\beta$ approx. 130 min; Figure 8 and Table 1). Notably, the extended circulatory half-life exhibited by the oversialylated version of rHuAChE coincides with the value predicted for maximal loading of rHuAChE with sialic acid residues. This value was calculated from the inverse linear relationship between circulatory residence and sialic acid-unoccupied glycan termini of rHuAChE expressed in HEK-293 cells [4].

Treatment of the rHuAChE *in vitro* with commercial α2,6ST also results in extended circulatory residence; the value obtained is very similar to that of rHuAChE produced by the α2,6ST-engineered HEK-293 cells, establishing that sialylation within the modified cells is highly efficient. The fact that the extended residence time now coincides with the predicted value for maximal sialic acid loading seemingly indicates that an upper limit for sialic acid incorporation into HEK-293-derived rHuAChE has been reached. However, determination of NeuAc suggests that the sialic acid content (approx. 5.5 sialic acid residues per rHuAChE subunit; Figure 3B) of the oversialylated versions of rHuAChE generated by the action of α2,6ST either *in vitro* or

in vivo is somewhat lower than that expected for complete sialic acid capping of biantennary and triantennary N-glycan termini. This observation raises the possibility that, although sialylation is highly efficient, a fraction of N-glycans remain refractive to sialylation. Furthermore HPAEC–PAD analysis revealed that some of the N-glycan termini of the oversialylated rHuAChE preparations are indeed only partly sialylated (Figures 6 and 7, and Table 2). This subset of glycan termini that are refractive to sialylation might serve as substrates for alternative clearance pathways and therefore might be responsible for the still relatively rapid clearance of oversialylated rHuAChE compared with native serum-derived forms of ChEs such as fetal bovine serum AChE.

When the recombinant bovine AChE [7], which differs from rHuAChE both in amino acid sequence (34 out of 583 residues divergence) as well as in the number of N-glycans (four N-glycans in rBoAChE; three N-glycans in rHuAChE), was expressed in HEK-293 cells, the resulting enzyme was cleared from the circulation faster than rHuAChE ($t_{\frac{1}{2}}\beta$ for rBoAChE was 29 min; $t_{\frac{1}{2}}\beta$ for rHuAChE was 80 min). In fact, the pharmacokinetics profile of rBoAChE was similar to that of a mutated version of rHuAChE that contained, like the bovine AChE, four utilized N-glycan sequons [7]. Nevertheless the sialylation of HEK-293-produced rBoAChE *in vitro* resulted in an extended circulatory residence, identical with that of oversialylated rHuAChE ($t_{\frac{1}{2}}\beta$ 130 min; see Figure 8C). This observation reinforces the idea that oligosaccharide processing, rather than the actual number of N-glycans or differences in amino acid sequence, is responsible for the differential pharmacokinetic behaviour of the various AChEs. Furthermore the fact that the two different recombinant glycoproteins generated by HEK-293 cells exhibit a similar extended circulatory residence after treatment with sialyltransferase suggests that the presence of sialylation-refractive glycans is imposed by a HEK-293 cell-dependent glycan-processing mode. The observation that not all N-glycan termini can serve as acceptors for sialic acid addition can be explained either by local constraints that do not allow access of sialyltransferase to AChE [30,46] or by the presence of N-glycan terminal structures that preclude NeuAc capping. Elucidation of the nature of the asialylated form of the N-glycans is currently under study.

Although sialic acid modulation improved the retention of both rHuAChE and rBoAChE, circulatory residence is still not as extended as that of native serum-derived cholinesterases. This suggests that other structural features of the carbohydrates as well as factors related to the protein itself (such as size/subunit assembly, surface charge and hydrophobicity) might have a role in determining the circulatory longevity of this protein type. For example, serum-derived cholinesterases are characteristically assembled into tetramers. In the past [4], the effect of oligomerization on clearance was examined by comparing the clearance rates of various rHuAChE preparations that differed in their relative amounts of monomers, dimers and tetramers. Our results failed to provide an indication of a role for oligomerization in determining circulatory residence. These studies were extended recently by monitoring the pharmacokinetic behaviour of a C-terminal truncated version of rHuAChE that cannot undergo subunit assembly. We found (results not shown) that this form of the enzyme has a clearance profile indistinguishable from that of the wild-type version of rHuAChE, suggesting that multimerization does not influence circulatory longevity. However, we note that these studies were performed in HEK-293 cells that were not genetically modified by α2,6ST; the undersialylated state of the enzyme products could therefore have masked the contribution of subunit assembly to circulatory residence.

Taken together, our observations suggest that sialylation extent is one of the important elements of a multifactorial mechanism for AChE clearance. Generation of a rHuAChE version displaying a pharmacokinetic profile similar to that of plasma-derived native cholinesterases might demand additional genetic manipulation or manipulation *in vitro*. However, identification of the required modifications would necessitate the prior elimination of the overriding effect exerted by undersialylation.

Importance of endogenous glyosyltransferases in cell lines used for high-level expression of recombinant proteins

A recently proposed mathematical model of glycoprotein sialylation in the Golgi compartment has suggested that, although cellular proteins are not expected to be undersialylated because of limited availability of sialyltransferase, biotechnological systems aimed at high production levels of recombinant proteins can give rise to undersialylated glycoproteins by virtue of overloading of the cellular glycosylation machinery with protein bulk [31]. In a recent study, an analysis of N-glycans associated with overexpressed secreted recombinant α-galactosidase A in CHO cells revealed that most of them were incompletely galactosylated and/or sialylated [47]. The authors suggested that the limiting step in recombinant glycoprotein overexpression in this cell system might be the relative inefficiency of *trans*-Golgi-residing glycosyltransferases. Here we show, by comparing sialylation levels of rHuAChE expressed in HEK-293 cell lines differing in their amounts of rHuAChE secretion (approx. 20-fold) that a higher content of sialic acid and a longer circulatory retention were indeed associated with low production levels of rHuAChE (Figure 4 and Table 1). An attempt to correct the undersialylation of rHuAChE expressed at high levels by alteration of its Golgi residence time (KDEL retention signal appendage to rHuAChE) did not result in an extended circulatory lifetime of the enzyme. However, by genetically engineering the cells to express a heterologous gene for α2,6ST, we have shown that it is possible to correct the relative paucity of sialyltransferase activity in the HEK-293 cells without affecting the yield of the recombinant product.

Because expression systems tailored for overproduction of recombinant proteins entail a concomitant compromise in post-translation modification efficiency, identification of the limiting factors and their provision by genetic modification might prove to be the method of choice for the establishment of production systems in which the protein of interest is adequately processed. Several reports have documented the use of the gene for α2,6ST to alter the properties of Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells, which lack the enzyme α2,6ST and therefore produce sugar chains terminating in the NeuAc-α2,3-Gal linkage [48–50]. In the present study we show that the 2,6ST oversialylation system *in vivo* could provide a useful approach for extending the circulatory residence of recombinant proteins that are cleared via removal pathways involving recognition of terminal galactose residues. Similar approaches might enable the promotion of circulatory longevity by modulating other limiting glycosyltransferases influencing pharmacokinetic performance.

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