Over-expression of a functionally active human G_{M2}-activator protein in *Escherichia coli*

Horst KLIMA,* Andreas KLEIN,* Gerhild VAN ECHTEN,* Günter SCHWARZMANN,* Kunihiko SUZUKI† and Konrad SANDHOFF*‡

*Institut für Organische Chemie und Biochemie, Gerhard-Domagk-Straße 1, D-5300 Bonn 1, Germany,
and †Brain and Development Research Center, University of North Carolina School of Medicine, Chapel Hill, NC 27599, U.S.A.

The cDNA of the human G_{M2}-activator protein was cloned into the expression vector pHX17. The plasmid encodes a fusion protein with a hexahistidine tail and a Factor Xa cleavage site at its N-terminus. The recombinant protein was purified from cell homogenates under denaturing conditions by metal-ion affinity chromatography in a single step and then was refolded. The hexahistidine tail could be removed when desired by digestion with Factor Xa. In a functional assay, the G_{M2}-activator thus generated from *Escherichia coli* and renatured, with or without the hexahistidine tail, was as active as the native G_{M2}-activator protein that was purified from human tissue. When added to the culture medium, the recombinant carbohydrate-free G_{M2}-activator, carrying the hexahistidine tail, could be taken up efficiently and restored the degradation of ganglioside G_{M2} to normal rates in mutant fibroblasts with the AB variant of G_{M2}-gangliosidosis, which is characterized by a genetic defect in the G_{M2}-activator protein. The prokaryotic expression system is useful for producing milligram quantities of a pure and functionally active G_{M2}-activator.

INTRODUCTION

The G_{M2}-activator is a lysosomal glycolipid-binding protein [1] and is an essential cofactor for in-vivo degradation of ganglioside G_{M2} by β-N-acetyl-D-hexosaminidase (EC 3.2.1.52; hexosaminidase A) [2]. Its genetic deficiency causes the abnormal accumulation of ganglioside G_{M2} in tissues of patients with the AB variant of G_{M2}-gangliosidosis. The protein is synthesized as a pro-protein that is processed to the mature glycoprotein of approx. 22 kDa. The protein has been sequenced by Edman degradation and consists of 162 amino acids [3]. A cDNA has been isolated from a human-fibroblast library that encodes the mature G_{M2}-activator [4]. Recently, the gene and two full-length cDNA clones of the G_{M2}-activator were isolated and characterized [5]. A mutation in the G_{M2}-activator gene has been identified in a patient with G_{M2}-gangliosidosis variant AB [6]. COS-1 cells transfected with an expression vector containing the G_{M2}-activator protein cDNA generated a functional G_{M2}-activator protein [7].

For structural and functional studies, large amounts of active protein are needed. While some eukaryotic expression systems, for example, baby-hamster-kidney (BHK) cells or insect cells infected with an appropriate recombinant baculovirus, can produce sufficient amounts of recombinant proteins, a suitable purification procedure must be developed for the proteins generated. Large amounts can be generated in *Escherichia coli* as well by subcloning the cDNA together with a strong promotor into a prokaryotic expression vector that is designed to facilitate the subsequent purification of the recombinant protein generated. An additional advantage is that *E. coli* has no endogenous G_{M2}-activator, which facilitates purifying and characterizing normal and mutant G_{M2}-activators. However, since the recombinant proteins produced by *E. coli* are not glycosylated, it is important to make sure that the G_{M2}-activator generated by such a prokaryotic expression system is functional.

In this report we describe the expression of an appropriate cDNA construct in *E. coli* to yield a carbohydrate-free human G_{M2}-activator fusion protein. This could be purified in a single step under denaturing conditions and could be renatured to yield a fully functional G_{M2}-activator. The unprocessed G_{M2}-activator was as active as the native activator isolated from human tissues in an *in-vitro* assay and could correct the metabolic defect of fibroblasts from a patient with G_{M2}-gangliosidosis AB variant when added to the culture medium.

EXPERIMENTAL

Materials

All chemicals were of the highest purity available commercially. Culture flasks (25 cm², 75 cm²) were from Falcon (Becton Dickinson and Co., Paramus, NJ). Picofluor 30 was from Packard (Groningen, The Netherlands). Restriction endonucleases, T4 DNA ligase and the T7 sequencing kit were obtained from Pharmacia (Freiburg, Germany). The expression plasmid pQE-9, the *E. coli* host M15, transformed with the plasmid pREP4, and the Ni^{2+}-nitrilotriacetic acid (NTA) agarose were purchased from Diagen (Hilden, Germany). Isopropyl β-D-thiogalacto-pyranoside (IPTG), the yeast extract, bacto-tryptone, minimal-essential medium (GIBCO no. 0410190 m) and *Taq* DNA polymerase were obtained from GIBCO-BRL (Eggenstein, Germany). The 'rainbow' protein standard was from Amersham (Braunschweig, Germany). The protease Factor Xa and gluta-thione were purchased from Boehringer (Mannheim, Germany). The preparation of reaction buffers and reaction conditions followed the recommendations of the suppliers in general. Oligonucleotides were synthesized on a Pharmacia 'Gene Assembler Plus'. Ganglioside G_{M1} was labelled in the sphingoid base using the method of Schwarzmann [8].

Construction of the expression plasmid pHX17 (Figure 1)

A new *Bam*HI restriction site and a cleavage site for the protease
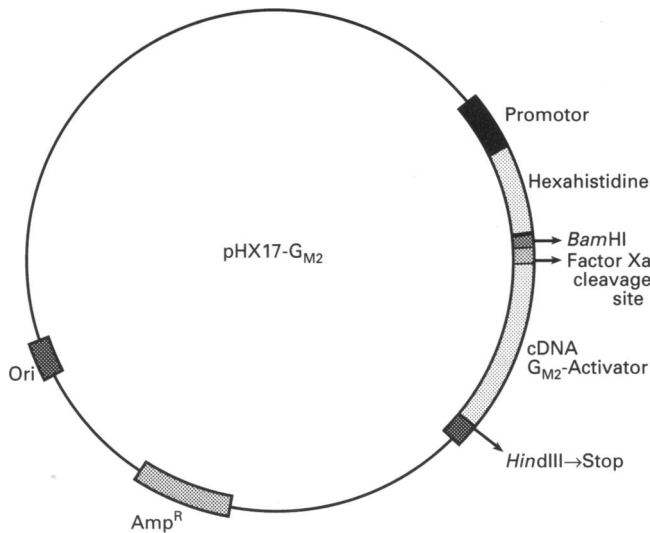

Figure 1   Construction and description of the expression vector pHX17

The vector was obtained from the plasmid pQE-9 by introducing a PCR-amplified cDNA construct containing the sequence for a Factor Xa-cleavage site and the mature G_{M2}-activator (see also Figure 2a). The plasmid pQE-9 belongs to the pDS-family [24] of plasmids and contains the regulatable promotor/operator element N250PSN250P29, which is repressed by the *E. coli lac* repressor but can be induced by IPTG, and the synthetic ribosomal-binding site (RBSII). It carries the gene for ampicillin resistance. The transcriptional terminator is from phage lambda.

Factor Xa (-Ile-Glu-Gly-Arg-Xaa-) were added on the 5′ side of the coding region of the mature G_{M2}-activator protein and a HindIII restriction site, re-creating the stop codon in the expression vector pQE-9 [9], was created at the 3′ end, by amplifying the G_{M2}-activator cDNA by PCR with suitable synthetic-oligo-nucleotide primers (Figure 2a). PCR amplification was performed using a Biometra thermocycler (Biometra, Göttingen, Germany). The amplified DNA was purified and digested with appropriate amounts of restriction enzymes. The host *E. coli*, M15/pREP4 [10], carrying the genes for kanamycin resistance and for the *lac* repressor on the plasmid pREP4 was made competent by treatment with $CaCl_2$. They were transformed with the ligation products (Figure 1) and were grown overnight on Luria broth (LB) plates containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml) at 37 °C [11]. The recombinant plasmid pHX17 was isolated and characterized by Southern blotting and DNA sequencing.

When induced by IPTG, this plasmid generates the mature human G_{M2}-activator protein fused to four amino acids encoded by the vector, six histidine residues followed by the Factor Xa-cleavage sequence on the amino terminus (Figure 2b).

Isolation and characterization of the plasmid DNA

Plasmid DNA was prepared using the method of Birnboim and Doly [12]. The plasmid DNA was purified further by chromatography on a column (Qiagen-tip 20, Diagen, Hilden, Germany) for sequencing. The insert was sequenced by the dideoxy chain-termination method [13] with internal oligonucleotides as primers, using the T7 sequencing kit and ^{35}S-dATP (Amersham, Braunschweig, Germany).

Purification of the recombinant G_{M2}-activator protein

E. coli cells were grown in 200 ml of S-medium (15 g·l⁻¹ bacto-

yeast extract, 25 g·l⁻¹ bacto-tryptone and 5 g·l⁻¹ NaCl) in a 1 litre flask with ampicillin (100 μg/ml) and kanamycin (50 μg/ml). At $A_{650} > 0.65$, expression was induced by adding IPTG to a final concentration of 3 mM. The cells were incubated for at least 6 h. The level of expression was monitored by analysing samples taken at intervals for the G_{M2}-activator protein by SDS/PAGE. The cells were harvested by centrifugation (4000 g, 4 °C, 15 min).The yield from 50 ml of the culture medium was 1.3 g of *E. coli*. Subsequent purification steps were carried out at room temperature. The cell pellet from a 50 ml culture was resuspended in 5 ml of extraction buffer (3 % v/v β-mercaptoethanol, 6 M guanidine hydrochloride and 0.1 M NaH_2PO_4, pH 8.0) for 1 h. After the extraction the cells were harvested by centrifuging (6000 g, room temperature, 15 min). The crude supernatant was loaded onto a column containing 3 ml of Ni²⁺-NTA agarose equilibrated with 11 ml of the extraction buffer. The recombinant G_{M2}-activator protein was purified by immobilized metal-affinity chromatography [14]. On an Ni²⁺-NTA adsorbent (Quiagen, Hilden, Germany) the metal ions are co-ordinated by six ligands. The hexahistidine residues of the recombinant G_{M2}-activator bind with high affinity to the Ni²⁺ ions to form a stable complex. The column was developed under mild conditions with a pH-step gradient (pH 8.0 loading step, pH 7.0 wash step and pH 5.0 elution of the product). The column was washed and was eluted sequentially with three column volumes of the extraction buffer, two volumes of the washing buffer (extraction buffer at pH 7.0) and two volumes of the elution buffer (extraction buffer at pH 5.0). All the fractions were collected in 2-ml samples. A sample of 100 μl from each fraction was dialysed against H_2O, analysed by SDS/PAGE and stained with Coomassie Blue [15].

Refolding of the recombinant G_{M2}-activator

The recombinant G_{M2}-activator in the elution buffer was diluted with five volumes of the folding buffer A [2 mM reduced glutathione, 0.2 mM oxidized glutathione, 50 mM Tris/HCl, pH 8.0 and 0.003 % (v/v) Tween 20] and stored at 4 °C for 18 h. The solution was dialysed against the folding buffer B [100 mM NaCl, 50 mM Tris/HCl, pH 8.0 and 0.003 % (v/v) Tween 20] for 16 h at room temperature. It was then dialysed for 16 h at room temperature against the Fx buffer (5 mM $CaCl_2$, 50 mM Tris/ HCl, pH 8.0 and 100 mM NaCl). The protein was tested for activity in an *in-vitro* G_{M2}-activator assay [16].

Digestion of the recombinant G_{M2}-activator with Factor Xa

Purified recombinant G_{M2}-activator was dialysed against the Fx buffer and was concentrated with Centricon-3 columns (Amicon, Witten, Germany). In a total volume of 80 μl, 120 μg of the recombinant protein was incubated with 1/10 the amount of the protease Factor Xa for 18 h at 37 °C. The reaction mixture was loaded onto an Ni²⁺-NTA column, equilibrated with the Fx buffer. The fraction that did not bind to the column was pooled and was assayed for G_{M2}-activator activity [16].

Other analytical methods

The protein content was determined using the method of Bradford [17] with BSA as a standard. SDS/PAGE was done according to the method of Laemmli [18].

Fibroblast culture and feeding studies

Human-fibroblast cell lines were grown in monolayers to confluence and were handled as described previously [19]. At 24 h

(a)

```
5'  1    A AGG CAC CTC TGC CGC CAC AGA CCT TGC AGT TAA CTC   37
aa  1                          Met Gln Ser Leu Met Gln        6
    38   CGC CCT GAC CCA CCC TTC CCG ATG CAG TCC CTG ATG CAG  76
aa  7    Ala Pro Leu Leu Ile Ala Leu Gly Leu Leu Leu Ala Thr 19
    77   GCT CCC CTC CTG ATC GCC CTG GGC TTG CTT CTC GCG ACC  115
                                Factor Xa
                        -BamHI- Ile Glu Gly Arg
                     5'c-gga-tcc-atc-gag-ggt-aga-agt-
aa  20   Pro Ala Gln Ala His Leu Lys Lys Pro Ser Gln Leu Ser 32
    116  CCT GCG CAA GCC CAC CTG AAA AAG CCA TCC CAG CTC AGT  154
         agc-ttt-tcc-tgg-gat 3'
aa  33   Ser Phe Ser Trp Asp Asn Cys Asp Glu Gly Lys Asp Pro 45
    155  AGC TTT TCC TGG GAT AAC TGT GAT GAA GGG AAG GAC CCT  193
aa  46   Ala Val Ile Arg Ser Leu Thr Leu Glu Pro Asp Pro Ile 58
    194  GCG GTG ATC AGA AGC CTG ACT CTG GAG CCT GAC CCC ATC  232
aa  59   Val Val Pro Gly Asn Val Thr Leu Ser Val Val Gly Ser 71
    233  GTC GTT CCT GGA AAT GTG ACC CTC AGT GTC GTG GGC AGC  271
aa  72   Thr Ser Val Pro Leu Ser Ser Pro Leu Lys Val Asp Leu 84
    272  ACC AGT GTC CCC CTG AGT TCT CCT CTG AAG GTG GAT TTA  310
aa  85   Val Leu Glu Lys Glu Val Ala Gly Leu Trp Ile Lys Ile 97
    311  GTT TTG GAG AAG GAG GTG GCT GGC CTC TGG ATC AAG ATC  349
aa  98   Pro Cys Thr Asp Tyr Ile Gly Ser Cys Thr Phe Glu His 110
    350  CCA TGC ACA GAC TAC ATT GGC AGC TGT ACC TTT GAA CAC  388
aa  111  Phe Cys Asp Val Leu Asp Met Leu Ile Pro Thr Gly Glu 123
    389  TTC TGT GAT GTG CTT GAC ATG TTA ATT CCT ACT GGG GAG  427
aa  124  Pro Cys Pro Glu Pro Leu Arg Thr Tyr Gly Leu Pro Cys 136
    428  CCC TGC CCA GAG CCC CTG CGT ACC TAT GGG CTT CCT TGC  466
aa  137  His Cys Pro Phe Lys Glu Gly Thr Tyr Ser Leu Pro Lys 149
    467  CAC TGT CCC TTC AAA GAA GGA ACC TAC TCA CTG CCC AAG  505
aa  150  Ser Glu Phe Val Val Pro Asp Leu Glu Leu Pro Ser Trp 162
    506  AGC GAA TTC GTT GTG CCT GAC CTG GAG CTG CCC AGT TGG  544
aa  163  Leu Thr Thr Gly Asn Tyr Arg Ile Glu Ser Val Leu Ser 175
    545  CTC ACC ACC GGG AAC TAC CGC ATA GAG AGC GTC CTG AGC  583
aa  176  Ser Ser Gly Lys Arg Leu Gly Cys Ile Lys Ile Ala Ala 188
    584  AGC AGT GGG AAG CGT CTG GGC TGC ATC AAG ATC GCT GCC  622
                                                   3'g-
aa  189  Ser Leu Lys Gly Ile ***                            193
    623  TCT CTA AAG GGC ATA TAA CAT GGC ATC TGC CAC AGC AGA  661
         aga-gat-ttc-ccg-tat-att-gca-acc
                        -HindIII- 5'
```

(b)

```
aa  1    Met Arg Gly Ser His His His His His His Gly Ser Ile  13
aa  14   Glu Gly Arg Ser Ser Phe Ser Trp Asp Asn Cys Asp Glu  26
aa  27   Gly Lys Asp Pro Ala Val Ile Arg Ser Leu Thr Leu Glu  39
aa  40   Pro Asp Pro Ile Val Val Pro Gly Asn Val Thr Leu Ser  52
aa  53   Val Val Gly Ser Thr Ser Val Pro Leu Ser Ser Pro Leu  65
aa  66   Lys Val Asp Leu Val Leu Glu Lys Glu Val Ala Gly Leu  78
aa  79   Trp Ile Lys Ile Pro Cys Thr Asp Tyr Ile Gly Ser Cys  91
aa  92   Thr Phe Glu His Phe Cys Asp Val Leu Asp Met Leu Ile  104
aa  105  Pro Thr Gly Glu Pro Cys Pro Glu Pro Leu Arg Thr Tyr  117
aa  118  Gly Leu Pro Cys His Cys Pro Phe Lys Glu Gly Thr Tyr  130
aa  131  Ser Leu Pro Lys Ser Glu Phe Val Val Pro Asp Leu Glu  143
aa  144  Leu Pro Ser Trp Leu Thr Thr Gly Asn Tyr Arg Ile Glu  156
aa  157  Ser Val Leu Ser Ser Ser Gly Lys Arg Leu Gly Cys Ile  169
aa  170  Lys Ile Ala Ala Ser Leu Lys Gly Ile ***             178
```

Figure 2 (a) Insert for the expression plasmid pHX17 and (b) amino-acid sequence of the recombinant G_{M2}-activator

In (a) the base sequence of the cDNA of the G_{M2}-activator sense strand is given with the amino-acid (aa) sequence deduced. The open-reading frame spans bp 59–641. The amino-acid sequence of the mature protein is from Ser^{-32} to Ile^{-193}. The N-glycosylation site is double underlined and the Factor Xa-cleavage site is underlined. The cDNA coding for the mature G_{M2}-activator [5] was amplified by PCR using the following oligonucleotides: sense primer (36 bp), 5'-C-GGA-TCC-ATC-GAG-GGT-AGA-AGT-AGC-TTT-TCC-TGG-GAT-3'; antisense primer (25 bp); 5'-CCA-ACG-TTA-TAT-GCC-CTT-TAG-AGA-G-3'; creating restriction sites for BamHI and HindIII.

after reaching confluency, the fibroblasts were washed three times with culture medium containing 0.3 % (v/v) heat-inactivated fetal-calf serum. Then 25 cm² culture flasks were incubated with 3 ml of medium containing 3 nmol [³H]G_{M1} per ml, labelled in the sphingoid base (specific activity, 4000 Ci mol^{-1}) and 0.3 % (v/v) heat-inactivated (30 min at 56 °C) fetal-calf serum under cell-culture conditions for 70 h. After incubation the cells were washed three times with 1 ml of PBS (140 mM NaCl, 3 mM KCl, 16 mM Na_2HPO_4 and 1.5 mM KH_2PO_4), detached by incubating with 2.5 ml of 0.25 % trypsin in PBS for 15 min at 37 °C and were isolated by centrifugation (1000 g, 4 °C, 10 min). The cell pellet was washed three times with 1 ml of PBS. This procedure removed all adsorbed glycolipids from the cell surface effectively but did not remove the inserted or intracellular glycolipids [20].

Incorporation of the G_{M2}-activator into fibroblast cells

The recombinant G_{M2}-activator or the human G_{M2}-activator, purified from tissues, was added to the [³H]ganglioside G_{M1}-containing medium (30 μg of human or of recombinant G_{M2}-activator per 3 ml). Cells from patients with G_{M2}-gangliosidosis variant AB (genetic G_{M2}-activator deficiency) and variant B (genetic β-hexosaminidase α-subunit deficiency) were maintained in the glycolipid- and the G_{M2}-activator-containing medium for 70 h under cell-culture conditions and then were harvested. The lipids were extracted from the cells as described below and were separated by t.l.c.

Extraction and t.l.c. of lipids

The cell pellets obtained from 25 cm² culture flasks were resuspended in 5 ml of chloroform/methanol/water (60:30:6, by vol.) and were extracted for 48 h at 50 °C. Aliquots of the cell extracts were mixed with Picofluor (20 ml) and the total cellular radioactivity was determined by scintillation counting. Insoluble material was removed by centrifugation (6000 g, 4 °C, 20 min). The supernatant was adjusted to pH 11 by adding NaOH (4 M). The solution was incubated for 16 h at 40 °C. After neutralization with acetic acid, the lipid extract was dried under N_2, resuspended in methanol/water/chloroform (47:48:3, by vol.) and loaded on RP-18 columns. The lipids were eluted from the columns with methanol followed by choloroform/methanol (1:1, by vol.). The fractions were pooled, applied to t.l.c. plates (Silica Gel 60, E. Merk, Darmstadt, Germany) and chromatographed with chloroform/methanol/0.22 % aqueous $CaCl_2$ (65:35:8, by vol.). Radioactive spots were visualized by fluorography [19].

RESULTS

A cDNA encoding the human G_{M2}-activator, with a hexahistidine tail and a Factor Xa cleavage site at its amino terminus, was cloned into the expression vector pHX17 and was expressed in E. coli M15. When induced by IPTG, production of the recombinant G_{M2}-activator could be observed already after 45 min and the maximum level was reached after 5 h. The product could be purified to near homogeneity by a single-step Ni^{2+}-NTA affinity column (Figure 3). The yield of the recombinant G_{M2}-activator protein was approx. 1.4 mg per 50 ml of the culture medium. However, the purified G_{M2}-activator was denatured due to the presence of 6M guanidine hydrochloride during the harvesting procedure and had to be renatured for activity. The renaturing

In (b) the recombinant protein consists of 178 amino acids. The recognition site for the protease Factor Xa in position 13 is double underlined. The sequence of the mature G_{M2}-activator protein is residues 17–178. The molecular mass of the recombinant protein is approx. 20 kDa.
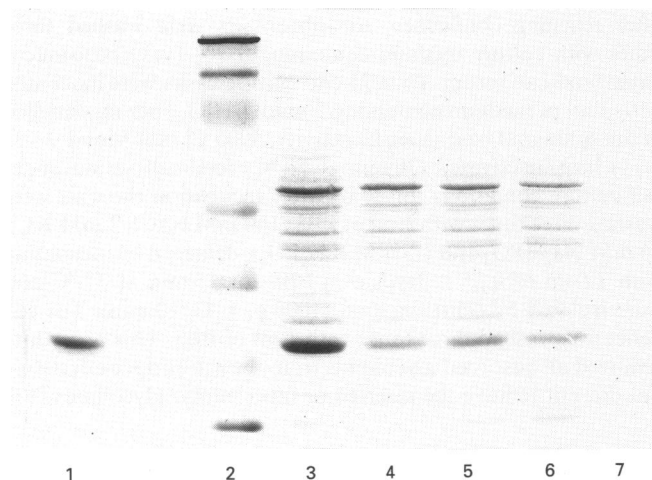
Figure 3   SDS/PAGE of the recombinant G$_{M2}$-activator obtained from *E. coli*

E. coli cells were collected by centrifugation from a 1-ml sample taken from the culture medium every 45 min to monitor the expression of the recombinant protein. The cells were boiled in a buffer containing 3% (w/v) SDS and 3% (v/v) β-mercaptoethanol. The denatured proteins were separated by SDS/PAGE and were stained with Coomassie Blue. Lane 1, recombinant G$_{M2}$-activator after the purification and refolding procedure; lane 2, protein standards (myosin 200 kDa, phosphorylase *b* 97.4 kDa, bovine serum albumin 69 kDa, ovalbumin 46 kDa, carbonic anhydrase 30 kDa, trypsin inhibitor 21.5 kDa and lysozyme 14.3 kDa); lane 3, 225 min after induction of expression; lane 4, 35 min after induction; lane 5, 90 min after induction; lane 6, 45 min after induction; lane 7, before addition of IPTG.

Table 1   Activity of the recombinant G$_{M2}$-activator protein in an *in-vitro* assay after refolding

The recombinant G$_{M2}$-activator containing the hexahistidine and the Factor Xa cleavage sequence at its amino terminus was purified under denaturating conditions and refolded. The protein was assayed in an *in-vitro* assay. The value given for the specific activity of the recombinant G$_{M2}$-activator is the mean of 19 independent measurements on different preparations of the recombinant G$_{M2}$-activator after refolding. An activator unit (AU) is defined by the amount of labelled G$_{M2}$ ganglioside cleaved under standard conditions; 1 AU = 1 nmol [^{3}H]G$_{M2}$/(unit β-hexosaminidase A per min) [25]. The native G$_{M2}$-activator refers to G$_{M2}$-activator purified from post-mortem tissues of Gaucher patients [25].

G$_{M2}$-activator	Specific activity (AU·mg^{-1})
Native	70.5
Recombinant	96.0 ± 16.0

procedure used was based on the method of Martson [21]. The protein was refolded by the step-wise dilution of guanidine hydrochloride and the slow oxidation of the cysteine groups to disulphide bonds by a glutathione-redox system. When assayed in the *in-vitro* system, the refolded recombinant G$_{M2}$-activator, with the six histidine residues and the Factor Xa cleavage sequence still attached to the N-terminus of the protein, was at least as active as the native G$_{M2}$-activator, purified from post-mortem tissues of Gaucher patients (Table 1). The hexahistidine residue could be removed by cleaving with Factor Xa and separating on an Ni^{2+}-NTA column. The G$_{M2}$-activator without
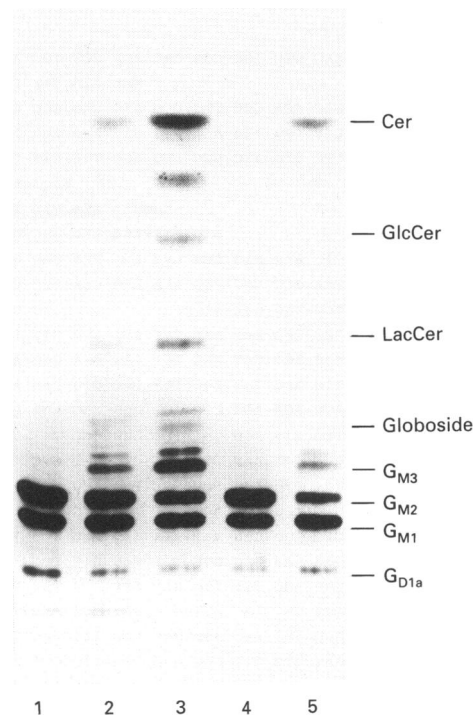

Figure 4   Metabolism of exogenously added [^{3}H]G$_{M1}$ in fibroblasts

Skin fibroblasts from normal controls and from patients with G$_{M2}$-gangliosidosis variants AB and B were cultured with [^{3}H]G$_{M1}$ labelled in the sphingosine moiety and with or without various preparations of G$_{M2}$-activator (30 μg/3 ml of culture medium each) in the medium for 70 h, then harvested and extracted. The lipids were separated by t.l.c. and the radioactive spots were visualized by fluorography. Lane 1, AB-variant cells; lane 2, AB-variant cells with the human G$_{M2}$-activator purified from the tissues of a Gaucher-disease patient; lane 3, AB-variant cells with the recombinant G$_{M2}$-activator; lane 4, B-variant cells with the recombinant G$_{M2}$-activator; lane 5, normal control cells. Abbreviations used: Cer, ceramide; Lac, lactose.

the histidine residues passed through the column, while that with the hexahistidine still attached was adsorbed by the column. The yield was approx. 30%. The G$_{M2}$-activator generated by this means had about the same specific activity as that with the hexahistidine tail. Therefore apparently the presence of the hexahistidine and of the Factor Xa-cleavage sequence did not affect the function of the G$_{M2}$-activator.

The recombinant G$_{M2}$-activator was detected in a Western blot (data not shown) using polyclonal antibodies that had been raised against purified G$_{M2}$-activator protein from human tissue, as described previously [22].

Cultured fibroblasts from patients with the AB variant of G$_{M2}$-gangliosidosis are deficient genetically in the G$_{M2}$-activator protein [1] and do not degrade exogenously added ganglioside G$_{M2}$. It has been demonstrated previously that the capacity to degrade G$_{M2}$ could be restored by adding purified G$_{M2}$-activator to the culture medium [19].

In our experiments, control fibroblasts could degrade [^{3}H]-ganglioside G$_{M1}$, labelled in the sphingoid moiety, added to the culture medium in a step-wise manner. The degradation was blocked, as expected, at the level of G$_{M2}$ in fibroblasts from patients with G$_{M2}$-gangliosidosis AB variant (Figure 4).

Adding either the recombinant carbohydrate-free G$_{M2}$-activator, with the hexahistidine tail still attached, or the native glycosylated G$_{M2}$-activator, purified from the post-mortem tissues of a Gaucher patient, to the culture medium of AB-variant cells eliminated the block on G$_{M2}$-ganglioside degradation completely.

Adding either of the G_{M2}-activators had no effect on the block of G_{M2} degradation in the B-variant fibroblasts (Figure 4). This finding was expected because the block in B-variant cells is due to a genetic defect in the β-hexosaminidase α-gene rather than the G_{M2}-activator gene and the B-variant cells contain the normal level of G_{M2}-activator. These observations are consistent with the findings *in vitro* and show that the recombinant G_{M2}-activator, without glycosylation and with the foreign hexahistidine and the Factor Xa cleavage sequence, is as active as the native G_{M2}-activator, under the same *in-situ* conditions.

DISCUSSION

The availability of the full-length cDNA coding for the human G_{M2}-activator protein opened up the possibility to study the details of the function of this protein in relation to the other reaction components, the β-hexosaminidase α- and β-subunits and the substrate, ganglioside G_{M2}, in more clearly defined systems. For these purposes the availability of large amounts of the G_{M2}-activator in a functionally active form is essential. The prokaryotic over-expression system described in this report has two major pragmatic advantages, the production of large quantities of the product and the simplicity with which a high degree of purification can be achieved. With a yield of nearly 30 mg per litre of bacterial culture, even a quantity of grammes may be feasible. The high-affinity binding of the hexahistidine residue to the Ni^{2+}-NTA column and its easy dissociation from the column allows effective purification to near homogeneity in a single step. A disadvantage of the system in our hands was that the recombinant G_{M2}-activator could not be obtained without being denatured. Attempts to obtain it in an active form, by destroying the membranes of the *E. coli* cells with a compression chamber ('mini-bomb') or by sonication, were unsuccessful.

Therefore the activator was extracted under strongly denaturing conditions in the presence of 6M guanidine hydrochloride and 3% (v/v) β-mercaptoethanol. The eight cysteine residues in the mature G_{M2}-activator can form four disulphide bonds that may be essential for its activity. The renaturation procedure described was devised after much trial and error and, while the procedure effectively renatures the G_{M2}-activator, different proteins are likely to require different renaturing conditions that must be defined empirically.

In the *in-vitro* assay [25], the recombinant and renatured G_{M2}-activator was consistently slightly more active than the native G_{M2}-activator purified from post-mortem tissues of Gaucher-disease patients. We interpret this to mean that the native G_{M2}-activator preparation, after many tedious purification steps, is still not as pure as the recombinant G_{M2}-activator after the single-step purification, or that the native activator is inactivated partially during the long purification procedure. We determined that the very low concentrations of detergent (0.003% v/v Tween 20) in the folding buffers did not affect the outcome of the *in-vitro* G_{M2}-activator assay.

Similarly the recombinant carbohydrate-free G_{M2}-activator was in every respect at least as effective as the purified native activator in restoring the G_{M2}-catabolizing capacity of cultured fibroblasts from patients with G_{M2}-gangliosidosis AB variant. This indicated that the G_{M2}-activator without carbohydrate chains could be taken up by these cells as efficiently as the glycosylated native G_{M2}-activator.

Initially we were apprehensive that the presence of foreign amino acids (four amino acids encoded by the vector, six histidines and four amino acids of the Factor Xa recognition sequence) and the lack of glycosylation might affect the function

of the G_{M2}-activator; however neither factor affected the activity. The activity was unchanged before and after the removal of the foreign amino acids, which can be removed with reasonable efficiency anyway, if the purpose and design of experiments make them undesirable. The high activity compared to the glycosylated native activator similarly confirmed that the carbohydrate chain is not essential for the G_{M2}-activator activity.

The equal effectiveness of the native and of the recombinant G_{M2}-activator in correcting the defective catabolism of the G_{M2}-ganglioside in the G_{M2}-gangliosidosis AB variant fibroblasts indicates that a mechanism that can take up the activator added to the culture medium exists that is independent of the carbohydrate chain. The carbohydrate-free G_{M2}-activator is quite likely to be bound to glycolipids at the cell surface and then taken up by endocytosis to the lysosome. However glycosylation is expected to be essential for the normal cellular function and for the routeing of the endogenously synthesized G_{M2}-activator to the lysosomes.

Another sphingolipid activator, *sap-B*, also retains its *in-vitro* activator function when deglycosylated, but a mutation that abolishes its glycosylation site is known to result in a *sap-B*-deficiency phenotype that resembles metachromatic leuko-dystrophy [23].

The prokaryotic over-expression system should be useful to isolate large amounts of functionally active G_{M2}-activator that could be used for X-ray crystallography, n.m.r. spectroscopy and for detailed studies of the enzymic reaction mechanism of G_{M2}-ganglioside hydrolysis.

REFERENCES

1 Sandhoff, K., Conzelmann, E., Neufeld, E. F., Kaback, M. M. and Suzuki, K. (1989) In The Metabolic Basis of Inherited Diseases 6th edn. (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.), pp. 1807–1839, McGraw-Hill, New York

2 Meier, E., Schwarzmann, G., Füst, W. and Sandhoff, K. (1991) J. Biol. Chem. **266**, 1879–1887

3 Fürst, W., Schubert, J., Machleidt, W., Meyer, H. E. and Sandhoff, K. (1990) Eur. J. Biochem. **192**, 709–714

4 Schröder, M., Klima, H., Nakano, T., Kwon, H., Quintern, L. E., Gärtner, S., Suzuki, K. and Sandhoff, K. (1989) FEBS Lett. **251**, 197–200

5 Klima, H., Tanaka, A., Schnabel, D., Nakano, T., Schröder, M., Suzuki, K. and Sandhoff, K. (1991) FEBS Lett. **289**, 260–264

6 Schröder, M., Schnabel, D. and Sandhoff, K. (1991) FEBS Lett. **290**, 1–3

7 Xie, B., McInnes, B., Neote, K., Lamhonwah, A. and Mahuran, D. (1991) Biochem. Biophys. Res. Commun. **177**, 1217–1223

8 Schwarzmann, G. (1978) Biochim. Biophys. Acta **529**, 106–114

9 Hochuli, E., Bannwarth, W., Döbeli, H., Gentz, H. and Stüber, D. (1988) Bio/Technology **6**, 1321–1325

10 Villarejo, P. and Zabin, I. (1974) J. Bacteriol. **120**, 466–474

11 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

12 Birnboim, H. C. and Doly, J. (1979) Nucleic Acids Res. **7**, 1513–1519

13 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467

14 Arnold, F. J., (1991) Bio/Technology **9**, 151–156

15 Merriyl, C. R. (1990) Methods Enzymol. **182**, 477–488

16 Conzelmann, E. and Sandhoff, K. (1987) Methods Biochem. Anal. **32**, 40–51

17 Bradford, M. (1976) Anal. Biochem. **72**, 248–254

18 Garfin, D. E. (1990) Methods Enzymol. **182**, 425–441

19 Sonderfeld, S., Conzelmann, E., Schwarzmann, G., Burg, J., Hinrichs, U. and Sandhoff, K. (1985) Eur. J. Biochem. **149**, 247–255

20   Schwarzmann, G., Hoffmann-Bleihauer, P., Schubert, J., Sandhoff, K. and Marsch, (1983) Biochemistry **22**, 5041–5048

21   Martson, F. (1987) in DNA Cloning: A Practical Approach, Vol. III (Glover, D. M., ed.), pp. 59–88, IRL Press, Oxford

22   Burg, J., Conzelmann, E. and Sandhoff, K. (1985) Ann. Hum. Genet. **49**, 41–45

23   Rafi, M. A., Zhang, X. L., De Gala, G. and Wenger, D. A. (1990) Biochem. Biophys. Res. Commun. **166**, 1017–1023

24   Bujard, H., Gentz, R., Lanzer, M., Stüber, D., Müller, M., Ibrahimi, I., Haeuptle, M.-T. and Dobberstein, B. (1987) Methods Enzymol. **155**, 416–433

25   Conzelmann, E. and Sandhoff, K. (1979) Hoppe-Seyler's Z. Physiol. Chem. **360**, 1837–1849