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Glycosylation plays a crucial role in glycoprotein stability and its correct folding. Murine acid sphingomyelinase (ASM) is a lysosomal glycoprotein. We studied the functional role of its individual N-linked oligosaccharides needed to maintain enzymatic activity and protein stability. Mutagenized cDNA constructs were heterologously expressed. All six potential N-glycosylation sites were modified. Incomplete glycosylation of the most distant C-terminal site resulted in two isoforms. Oligosaccharides at N-84, N-173, and N-611 were found to be of minor importance for enzymatic activity. The glycosylation defect at N-333 or N-393 reduced the enzymatic activity to 40% and at N-518 to less than 20%. These mutations did not effect the K_m value. Glycosylation at N-333 and N-393 mainly contributed to the enzyme stability and prevented degradation at lysosomal acidic pH, whereas the low residual enzymatic activity of mutant ASM deficient in glycosylation at N-518 was caused by protein misfolding. The mutant protein was also prone to proteolysis when trapped in the endoplasmic reticulum/cis-Golgi after brefeldin A application. Insufficiently glycosylated ASM formed a stable complex with BiP, an immunoglobulin heavy chain-binding protein, and thus remained in the endoplasmic reticulum. $^{32}PO_4$ labeling revealed that the glycosylation mutants of ASM were phosphorylated predominantly at mannose residues of oligosaccharides linked to N-84, N-333, and N-393.

Acid sphingomyelinase (ASM,[1] E.C. 3.1.4.12) is an ubiquitous lysosomal hydrolase that cleaves sphingomyelin to ceramide and phosphocholine. In 1987, Quintern *et al.* (1) purified human acid sphingomyelinase from urine to homogeneity. Subsequent discovery of the primary structure enabled functional expression in an eucaryotic cell culture system (2).

Deficient or reduced ASM activity causes sphingomyelin accumulation known in humans as Niemann-Pick disease sphingolipidosis. Depending on the amount of residual enzymatic activity, the neurovisceral type A or the less severe visceral type B of this autosomal recessive disease is observed (3).

Recently, several mutations in the *ASM* gene of Niemann-Pick disease patients have been reported, leading to an enzyme of no or drastically reduced activity. Transgenic mice with a disrupted *ASM* gene exhibit a phenotype comparable to that of Niemann-Pick disease, type A, patients. The neurological symptoms of these mice are accompanied by the loss of Purkinje cells as revealed by histological studies (4, 5).

Besides the catabolic function of this lysosomal enzyme, involvement of ASM in the ceramide-dependent signal transduction pathway has been discussed controversially (6–9). As a lysosomal hydrolase, ASM undergoes several posttranslational processing and targeting steps. The N-terminal signal sequence directs the primary translation product into the ER. The signal sequence, which has a length of approximately 45 amino acids for human as well as murine ASM according to the von Heijne prediction (10), is cleaved during this process. In the ER, oligosaccharides are transferred to certain Asn residues and further modified in the Golgi apparatus.

The oligosaccharide chains may share a variety of different functions. They are known to contribute to the proper protein folding and to preserve biological activity by stabilizing the protein conformation and protecting against proteolytic degradation (11–13). Furthermore, oligosaccharides are important for correct targeting of lysosomal proteins. To that end, certain mannose residues are phosphorylated in position 6 by a two-step reaction in the ER and the Golgi apparatus. Such tagged proteins bind to the mannose-6-phosphate receptor in the *trans*-Golgi network and are transported by coated vesicles to the lysosomes.

The aim of the present study was to characterize the functional aspects of the individual oligosaccharides of murine ASM. The enzyme can easily be expressed in a eucaryotic cell system and assayed. ASM activity is particularly stable and seems to be independent of further components. Therefore, it represents a useful model to investigate the various functions of glycosylation.

Since ASM is highly conserved between humans and mice, most of the results obtained for the murine protein should be valid also for the human enzyme (14).

EXPERIMENTAL PROCEDURES

Reagents—Tissue culture medium and reagents were purchased from Seromed (Berlin, Germany) and Life Technologies, Inc., and the radiochemicals were from Amersham Corp. Restriction enzymes and G418 were obtained from Life Technologies, Inc. Nitrocellulose membranes and P81 membranes used were from Schleicher & Schuell and Whatman, respectively. Brefeldin A, kanamycin, tunicamycin, Freund's adjuvant, and protein A-Sepharose were purchased from Sigma. Endoglycosidases were obtained from Boehringer Mannheim. The pRc/CMV vector was bought from Invitrogen, and the T7 sequencing kit and the USE site-directed mutagenesis kit were from Pharmacia Biotech Inc. Anti-BiP antiserum was purchased from Stressgen and Cy3 conjugated anti-rabbit-IgG antibody from Jackson. Glycerol gelatin was obtained from Merck.

Site-directed Mutagenesis—Murine ASM cDNA (14) was cloned into

[1] The abbreviations used are: ASM, acid sphingomyelinase; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; BiP, immunoglobulin heavy chain-binding protein; CMV, cytomegalovirus; Endo, endo-β-N-acetylglucosaminidase; PNGase, N-glycosidase; HEK, human embryonic kidney.

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the *Eco*RI site of puc13 (Fig. 1). Using the unique site elimination kit, each individual Asn codon of the six canonical *N*-glycosylation site (Asn-Xaa-Ser/Thr) was converted to a Gln codon.

Multiple mutations were introduced by exchange of cDNA cassettes using the restriction sites indicated in Fig. 1. Each mutated cDNA was controlled by DNA sequencing (T7 sequencing kit). Wild-type and mutant cDNAs were cloned into the *Hin*dIII restriction site of the pRc/CMV vector.

ASM Antibody—The full-length coding sequence of murine ASM cDNA without the 5′ terminal part was cloned into the pET8c vector (15). Expression in Bl21(DE3)pLysS *Escherichia coli* yielded a polypeptide representing the amino acids 100 to 627 of the murine ASM in abundant amounts. The protein was first separated by SDS-polyacrylamide gel electrophoresis and eluted with a minimal volume of 0.1% SDS, 100 mM Tris-Cl (pH 8). It was then precipitated with four volumes of acetone and dissolved in PBS. Polyclonal anti-ASM serum was raised in rabbits by an injection of 200 μg of recombinant protein in combination with Freund's complete adjuvant followed by three booster injections of 100 μg of protein in Freund's incomplete adjuvant in 2-week intervals. Serum was obtained 5 days after final injection.

Cell Culture and Transfection—HEK293 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. A suspension of approximately 2×10^6 cells was electroporated in culture medium at 450 V, 250 μF in the presence of 15 μg/ml circular plasmid DNA (pRc/CMV constructs) for transient or 1 μg/ml *Sca*I linearized plasmid DNA for stable expression (electroporator and 0.4-cm electroporation cuvettes by Bio-Rad). Transiently expressing cells were cultured for 48 h prior to harvest. Stable expressing cells were plated after electroporation in various dilutions on 100-mm dishes. After 24 h, the medium was supplemented with G418 at a concentration of 450 μg/ml. After 2 weeks of selection, individual cell clones were picked, expanded, and tested for expression by Northern blotting and enzymatic assays of ASM or neomycin phosphortransferase.

Metabolic Labeling and Immunoprecipitation—Transfected HEK293 cells grown to confluency in 60-mm dishes were starved in 1 ml of either methionine-free modified Eagle's medium or PO_4-free Dulbecco's modified Eagle's medium for 1 h prior to pulse labeling with 150 μCi L-[^{35}S]methionine or 250 μCi [^{32}P]orthophosphoric acid for the indicated periods of time. In case of the pulse-chase experiment, cells were incubated for an additional 2 h with normal cell culture medium. After metabolic labeling, cells were washed with PBS, harvested, and extracted in 200 μl of extraction buffer (0.1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, and 20 mM Tris-Cl, pH 7.2). For extraction of $^{32}PO_4$-labeled cells, the buffer also contained 5 mM NaF and 1 mM Na$_3$VO$_4$. Extracts were centrifuged for 15 min at $100,000 \times g$ and then incubated for 1 h with anti-ASM antiserum in a 1:60 dilution and subsequently treated for 1 h with 0.5 volume of protein A-Sepharose diluted 1:10 in extraction buffer. BiP precipitation was carried out using a monoclonal mouse anti-BiP antiserum in a dilution of 1:200. Immunoprecipitated material was washed with three changes of 1% Nonidet P-40, 20 mM Tris-Cl, pH 7.2. All steps were performed at 4 °C. Immunoprecipitations from culture medium were performed with 800 μl of medium cleared by ultracentrifugation. Immunoprecipitated material was resuspended (4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.05% bromphenol blue, 0.125 M Tris-Cl, pH 6.8) and separated on a 10% SDS-polyacrylamide gel. The gel was dried and exposed to x-ray film. The gel was analyzed by a PhosphorImager (Molecular Dynamics) when quantitative comparison of the labeled protein was necessary. Tunicamycin or brefeldin A was added at 10 or 2 μg/ml, respectively, to the cells 1 h prior to the labeling. Digestion with Endo F/PNGase F was performed with the immunoprecipitated material resuspended in 20 μl of extraction buffer for 2–3 h at 37 °C using 0.1 unit. For Endo H digestion, the immunoprecipitated material was resuspended in 20 μl of acidic buffer (30 mM sodium acetate, pH 5, 10 mM mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 0.02% SDS) and incubated for 13 h at 37 °C using 0.002 unit.

Immunofluorescence Microscopy—About 10^5 embryonal fibroblasts from ASM-deficient mice (4) at passage six were suspended in cell culture medium, electroporated at 500 μF and 450 V with 15 μg/ml plasmid DNA, and plated as duplicate sets on 60-mm dishes for 48 h. The cells were washed with PBS and fixed with freshly prepared acetone:methanol (1:1) at room temperature for 2 min. After washing with PBS, the fixed cells were incubated with 3% bovine serum albumin and a 1:200 dilution of rabbit anti-ASM antiserum in PBS for 1 h. After washing with three changes of 0.5% Triton X-100 in PBS over 10 min, the cells were exposed to a 1:800 dilution of anti-rabbit-IgG antibody conjugated with CY3 in 3% bovine serum albumin/PBS for another hour. Cells were washed as described above, mounted with glycerol
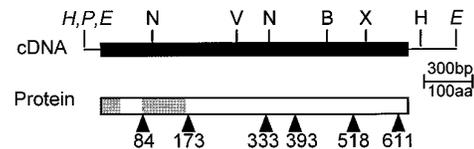

FIG. 1. **Schematic presentation of murine ASM cDNA and protein.** An open reading frame of 1881 base pairs (*black bar*) codes for a protein of 627 amino acid residues. Position of restriction enzyme sites (*H, Hin*dIII; *P, Pst*I; *E, Eco*RI; *N, Nco*I; *V, Eco*RV; *B, Bgl*II; *X, Xho*I) used for cloning are indicated. Italic letters represent sites of the used cloning vector. Signal peptide (amino acids 1–44) and saposin-like domain (amino acids 84–164) are marked as *gray boxes*. Position of the canonical *N*-glycosylation sites are shown by *arrowheads* with the amino acid number of the respective asparagine.

gelatin and coverslips, and viewed with a microscope equipped for epifluorescence (Zeiss). For BiP staining, a polyclonal rabbit anti-BiP antiserum in a dilution of 1:200 was used as a first antibody.

Enzymatic Assays—Transfected HEK293 cells were washed with PBS and harvested. To determine the ASM activity, the cells were lysed in extraction buffer (0.1% Nonidet P-40, 20 mM Tris-Cl, pH 7.2). Extracts were assayed as described previously (1) using ^{14}C-labeled sphingomyelin (16) with a specific radioactivity of 8000 dpm/nmol. Protein concentrations were determined as described previously (17) using bovine serum albumin as reference. To determine the neomycin phosphotransferase activity, cells were extracted in detergent-free buffer and incubated with kanamycin and [γ-^{32}P]ATP as described previously (18). Phosphorylated proteins were removed from the phosphorylated kanamycin by filtration of the incubation products through two layers of nitrocellulose membrane and two layers of P81 membrane using a filtration manifold (19). The amount of labeled kanamycin on the P81 membrane was quantified with a PhosphorImager (Molecular Dynamics) and served as a relative measurement of neomycin phosphortransferase activity in the cell extracts.

RESULTS

N-Glycosylation Is Essential for Murine ASM Activity—The cDNA of murine ASM (14) was cloned into the eucaryotic expression vector pRc/CMV to characterize the glycosylation. The minigene is under the control of the CMV promoter. Plasmid DNA was transfected either transiently or stably in HEK293 cells. Because cDNA-derived murine ASM contains six potential N-glycosylation sites at positions N-84, N-173, N-333, N-393, N-518, and N-611, each of these sites was eliminated by exchanging the codons for asparagine to glutamine in the cDNA using site-directed mutagenesis (ΔGS1 to ΔGS6, respectively). Combinations of these mutations were obtained by exchanges of cDNA cassettes using the restriction sites shown in Fig. 1. Mutant ASM cDNAs were subsequently cloned and expressed under the same conditions as the wild type.

The importance of the *N*-linked oligosaccharides for the maintenance of ASM enzymatic activity was evaluated. Extracts of lysed cells producing recombinant ASM were treated with Endo F/PNGase F. This treatment led to a loss of the activity compared to the control incubated without glycosidases. The complete enzymatic deglycosylation was demonstrated by immunoprecipitation after pulse labeling cells for 3 h with [^{35}S]methionine (Fig. 2*A*).

Wild-type murine ASM yielded two distinct polypeptides of 70 and 72 kDa, which were reduced in size to a single ~60-kDa protein upon deglycosylation with Endo F/PNGase F. This digestion product is completely deglycosylated because its molecular mass is identical to that of the mutant ASM ΔGS(1–6) with all putative *N*-glycosylation sites deleted.

The two distinct polypeptides visible after wild-type ASM immunoprecipitation do not result from successive processing steps during the pulse labeling as they proved to be time-independent in a pulse-chase experiment (Fig. 2*B*). Therefore, a variability in the use of the *N*-glycosylation sites must be the reason for this mass heterogeneity.

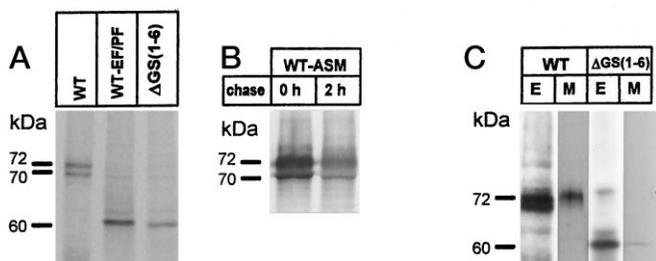A significant portion of ASM activity is released into the cell

FIG. 2. **Glycosylated and deglycosylated murine ASM.** HEK293 cell clones overexpressing wild-type (*WT*) and mutant ASM (ΔGS(1–6)) were pulse-labeled with [³⁵S]methionine and separated on 10% SDS-polyacrylamide gel electrophoresis and analyzed by fluorography. In *A,* cells were labeled for 3 h, and immunoprecipitated material was treated with 0.1 units Endo F/PNGase F (*EF/PF*) for 2 h. In *B,* cells were labeled for 1 h, followed by a chase period of 0 or 2 h prior to immuno-precipitation. In *C,* cells were labeled for 5 h. Cell extract (*E*) and culture medium (*M*) were subjected to immunoprecipitation. Proteins isolated from the medium were fluorographed 10 times longer than proteins from cell extracts.

culture medium upon overexpression of wild-type protein. This activity is associated with a protein slightly larger than the cellular form as determined by immunoprecipitation. The difference in protein mass is most likely due to a further modification of oligosaccharides during the secretion process because this size difference disappears for the nonglycosylated mutant ΔGS(1–6) (Fig. 2*C*).

Nonglycosylated ASM has been demonstrated to be inactive. We, therefore, determined the enzymatic half-life of this lysosomal protein by the administration of tunicamycin. Tunicamycin inhibits *N*-glycosylation and consequently stops the supply of newly translated functional ASM. The decay of the residual lysosomal enzyme activity of ASM in cell extracts revealed a half-life of about 14 h (Fig. 3).

Each Potential N-Glycosylation Site Is Modified with Oligosaccharides—Wild-type ASM and glycosylation site-deficient mutants were expressed transiently in HEK293 cells, and resulting ASM proteins were immunoprecipitated after 4 h pulse labeling with [³⁵S]methionine. Wild-type ASM resulted in two protein bands of 70 and 72 kDa. The loss of every single oligosaccharide group of the mutated ASMs shifted the double band by 2 kDa to a lower molecular mass. Only mutant ΔGS6 that lacked the most C-terminal glycosylation site yielded a single predominant translation product, which corresponded in molecular mass to the lower band of wild-type ASM (Fig. 4*A*). These results indicated that each of the six canonical sites is in fact glycosylated in the wild-type enzyme. A partial usage of the sixth site (N-611) appears to cause the observed mass heterogeneity in wild-type and in ΔGS1 to ΔGS5.

These observations were further confirmed by the investigation of a series of multiple mutated constructs with a decreasing number of functional glycosylation sites. Transient expression, [³⁵S]methionine pulse labeling, and subsequent immunoprecipitation yielded proteins of a stepwise decreasing size due to the successive loss of oligosaccharide chains. Complete removal of all canonical glycosylation sites resulted in a single polypeptide of 60 kDa. This molecular mass is identical to that obtained by treatment of wild-type ASM with PNGase F/Endo F or by expression of wild-type ASM in HEK293 cells in the presence of tunicamycin, a drug inhibiting *N*-linked glycosylation (Fig. 4*B*).

To investigate the type of the oligosaccharide structure, wild-type and mutant ASM were subjected to Endo H degradation. Proteins were immunoprecipitated from stably overexpressing HEK293 cell clones after 2 h of [³⁵S]methionine pulse labeling and a subsequent chase period of 4 h to ensure completion of oligosaccharide processing. In each case, Endo H digestion
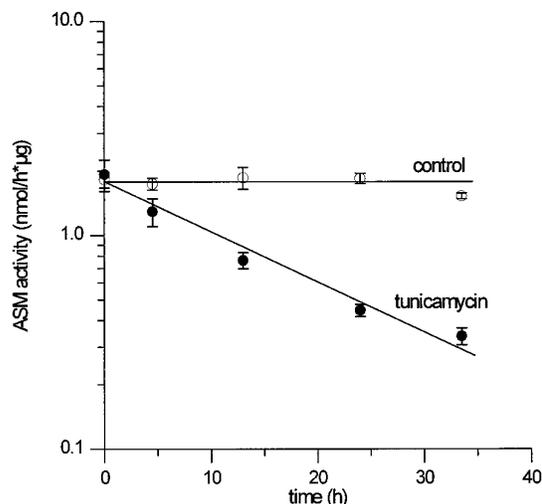

FIG. 3. **Time-dependent decay of cellular ASM activity after tunicamycin administration.** Half-confluent HEK293 cells overexpressing ASM were subjected to 4 μg/ml tunicamycin. After various periods of incubation, cellular proteins were extracted, and ASM activity and total extracted cell protein were determined. Control values were obtained by analogous treatment without tunicamycin. Each data point is based on a set of three independent experiments. The logarithmic scale of the specific activity shows an exponential decay of residual cellular ASM activity with a half-life of 14 h. *Bars,* S.D.

resulted in a protein band of 60 kDa corresponding to the completely deglycosylated form, which shows that biantennary complex type oligosaccharide structures are not present in murine ASM expressed in HEK293 cells (Fig. 4*C*).

N-linked Oligosaccharides at N-333, N-393, and N-518 Are Essential for Enzymatic Activity—HEK293 cells were transfected with wild-type ASM and the mutant constructs ΔGS1 to ΔGS6. Their ASM activity was assayed. Under the chosen conditions for transfection, cell culture, and extraction, an increase of ASM activity by about 0.2 μmol/h per mg of total cell protein was measured for the wild-type construct.

To compensate for variations in the transfection efficiency between the different ASM expression constructs, the neomycin phosphotransferase activity resulting from the neo-resistance gene of the pRc/CMV vector was used as a reference. The residual activity of the various mutant ASMs relative to the wild-type ASM are shown in Fig. 5*A*. Only oligosaccharides at N-333, N-393, and N-518 seemed to be of major importance for the enzymatic activity. Single elimination of the other sites (constructs ΔGS1, ΔGS2, and ΔGS6) only slightly reduced the activity. However, constructs ΔGS3 and ΔGS4, lacking glycosylation at N-333 and N-393, respectively, exhibited only 40% of wild-type activity, whereas construct ΔGS5, altered in N-518, possessed less then 20% residual activity.

The influence of these three glycosylation sites on the enzymatic activity was further investigated. The K_m values of all three mutant enzymes were that of the wild-type level of 25 μM.

A characteristic loss of ASM activity in extracts from overexpressing cells was observed after preincubation for 20 h at pH 4.5 and 37 °C prior to the enzymatic assay. Wild-type ASM activity was reduced to 43%, which is in good agreement with the previously determined *in vivo* half-life of the enzymatic activity of 14 h. Although the mutant enzymes ΔGS1, ΔGS2, and ΔGS6 behaved in the same manner as the wild-type enzyme, ΔGS3 and ΔGS4 showed a severe loss and ΔGS5 showed a moderate loss compared to the wild-type enzyme during this preincubation (Fig. 5*B*). The low but quite stable residual activity of ΔGS5 might refer to an incorrect protein folding. This interpretation is further supported by the immunofluorescence and brefeldin A experiments, (Figs. 7 and 8).
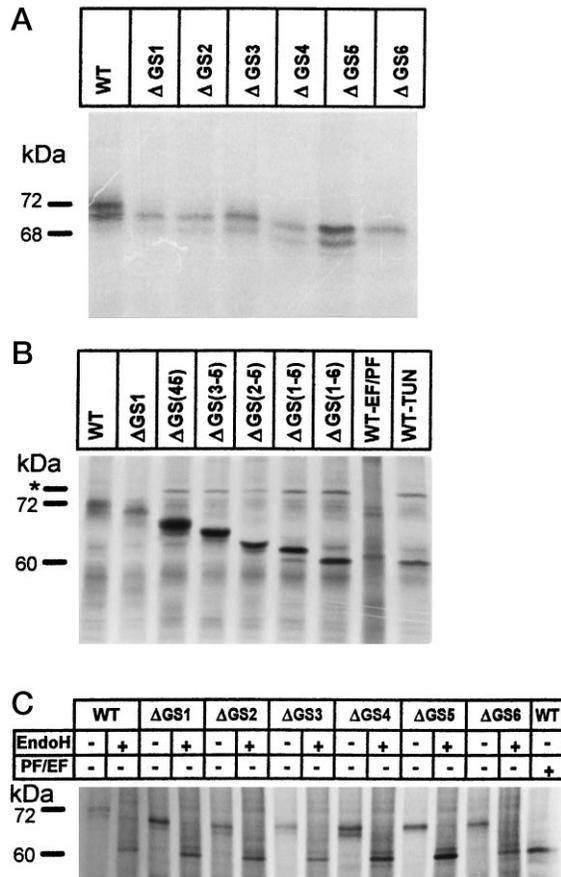
FIG. 4. **Individual glycosylation of murine ASM.** In *A* and *B*, HEK293 cells transiently expressing wild-type and mutant ASM were labeled with [^{35}S]methionine for 4 h. In one case, cells were treated with 10 μg/ml tunicamycin (*TUN*) prior to and during labeling. Cellular proteins were extracted and immunoprecipitated. In one case, precipitated material was deglycosylated with 0.1 units Endo F/PNGase F for 2 h (*EF/PF*). The *asterisk* indicates a protein of approximately 80 kDa (BiP) that coprecipitated with some incompletely glycosylated ASM mutants. In *C*, HEK293 cell clones overexpressing wild-type and mutant ASM were labeled with [^{35}S]methionine for 2 h prior to a chase period of 4 h. Immunoprecipitated material was treated with 0.002 unit of Endo H for 13 h or 0.1 units of Endo F/PNGase F for 3 h.

To test if this loss of activity is caused by protein degradation, HEK293 cell clones overexpressing wild-type and mutant ASM were pulse labeled with [^{35}S]methionine. The ASM proteins were immunoprecipitated from the cell extracts either with or without previous application of the acidic preincubation (Fig. 5, *C* and *D*). Neither the amount nor the size of wild-type ASM appeared to be influenced by the preincubation. We conclude that the observed decrease in activity results from conformational changes. The protein amount of mutant ASM ΔGS3 and ΔGS4, however, decreased by approximately 50% during preincubation, indicating an elevated sensitivity to proteases. The severe loss of enzymatic activity of these two mutants cannot be explained exclusively by the reduction in the protein level. Therefore, we assume an additional increase in conformational lability.

The degree of protein degradation of mutant ASM ΔGS5 was significantly higher than the concomitant loss of enzymatic activity (see "Discussion"). The mutant ASM in which all three crucial glycosylation sites at N-333, N-393, and N-518 are eliminated was totally inactive, and the polypeptide was nearly completely degraded during the described preincubation.

Elimination of Defined N-Glycosylation Sites Causes Stable Association to BiP—In the experiment shown in Fig. 4*B*, a protein of about 80 kDa was found to coprecipitate with the
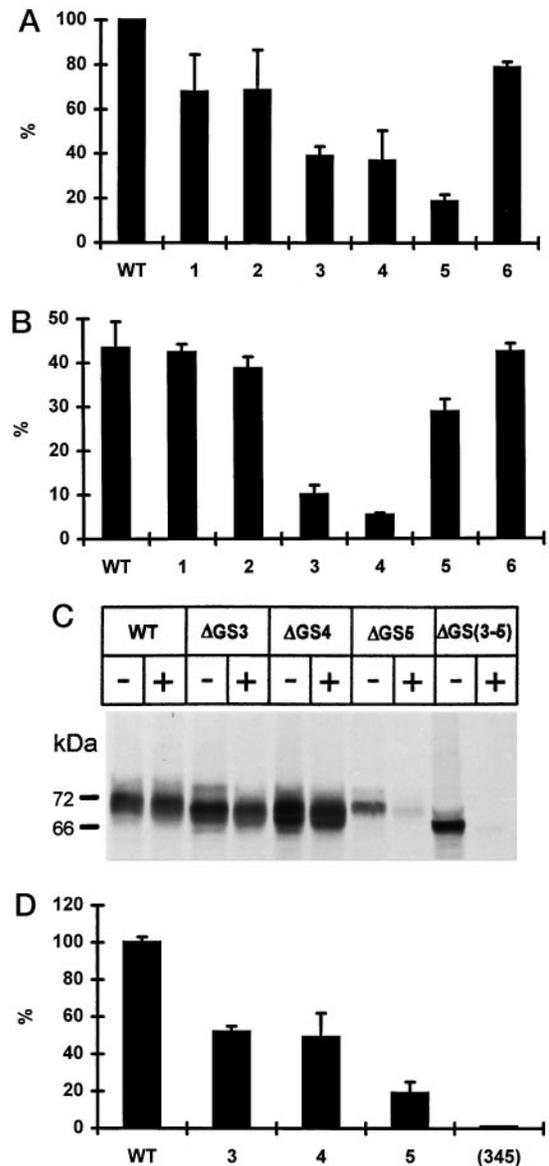

FIG. 5. **Residual enzymatic activity of mutant ASM deficient in individual glycosylation sites.** In *A*, HEK293 cells were transfected with ASM expression constructs and mock-transfected with pRc/CMV vector. Activities exceeding the endogenous level were normalized by the coexpressed neomycin phosphotransferase activity. The activities of the mutant ASM proteins, ΔGS1 to ΔGS6 (indicated as *WT* and *1–6*, respectively), obtained from three independent sets of experiments are expressed in percentages of the wild-type level. The *columns* represent the relative ASM activities measured immediately after protein extraction. *Bars*, S.D. In *B*, extracts of HEK293 cells transiently expressing wild-type and mutant ASM were assayed prior to and after incubation at pH 4.5 and 37 °C for 20 h. The *columns* represent the residual activity of the individual ASM constructs after this preincubation in percentages of their starting values. *Bars*, S.D. In *C*, protein extracts of HEK293 cell clones overexpressing wild-type and mutant ASMs labeled with [^{35}S]methionine for 3 h were subjected to a preincubation of 20 h at pH 4.5 and 37 °C prior to immunoprecipitation. In *D*, quantification of the relative loss of protein during this preincubation using a PhosphorImager and the ImageQuant software. *Bars*, S.D.

ASM in case of mutants in which at least two oligosaccharides (ΔGS(4+5) and further) have been eliminated. Wild-type ASM produced in the presence of tunicamycin also showed a stable association to this protein. In contrast, wild-type ASM and mutants with only one missing oligosaccharide group did not form a complex with this protein, as shown in Fig. 4*A*. Ig heavy chain binding protein (BiP, grp78) is known to assist in correct glycoprotein folding in the ER (20, 21). Several insufficiently
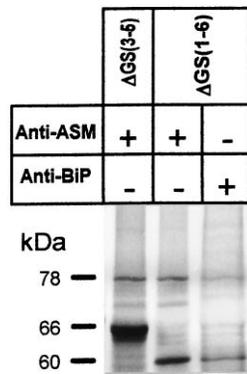
FIG. 6. **Stable association of insufficient glycosylated ASM to BiP.** HEK293 cells transiently expressing mutant ASMs were labeled with [^{35}S]methionine for 4 h and immunoprecipitated either with anti-ASM or anti-BiP antibodies. BiP coprecipitated with anti-ASM antibodies and *vice versa*.

FIG. 7. **Effect of brefeldin A on overexpression of ASM.** HEK293 cell clones overexpressing wild-type and mutant ASM were treated with 2 μg/ml brefeldin A prior to and during pulse labeling with [^{35}S]methionine for 3 h.

glycosylated glycoproteins have been reported to associate stably with BiP and are retained in the ER (22–25). The identity of BiP and the protein associated with several incompletely glycosylated ASM mutants was demonstrated by immunoprecipitation, as shown in Fig. 6. Labeled cell extracts of HEK293 cells transiently expressing completely deglycosylated ASM (ΔGS(1–6)) were incubated with antibodies to either ASM or BiP. Both antibodies precipitated the 60-kDa polypeptide of the unglycosylated ASM as well as the 78-kDa protein corresponding to BiP.

The important role of the glycosylation site at N-518 is further supported by the experiment shown in Fig. 7. Wild-type and several mutant ASMs were immunoprecipitated from the corresponding [^{35}S]methionine pulse-labeled cell clones after administration of brefeldin A. Brefeldin A is a drug known to inhibit the vesicular transport through the Golgi apparatus (26). Except for ΔGS5, all tested ASM variants were slightly reduced in size and accumulated when trapped in ER/*cis*-Golgi. Apparently, the trapped protein undergoes an intense oligosaccharide trimming. The increase in protein amount is probably due to the inhibited secretion because no ASM protein was found in the cell culture medium of overexpressing cells after brefeldin A treatment (data not shown). However, in the case of construct ΔGS5, mutant protein is hardly detectable when expression was performed in the presence of brefeldin A. This indicated that the incorrectly folded glycoprotein is immediately degraded when retained in ER/*cis*-Golgi.

The stable complex of incompletely glycosylated ASM to BiP is retained in the ER, as demonstrated by immunofluorescence microscopy. We overcame background problems by the ubiquitously expressed endogenous ASM by using embryonal fibroblasts of ASM-deficient mice for transient transfection (4). Immunofluorescence microscopy revealed the accumulation of wild-type ASM in the lysosomes but localization of ASM ΔGS(3–5) and of BiP in the ER (Fig. 8). Also ΔGS3 and ΔGS4 were found in lysosomes, but considerable amounts of ΔGS5 were retained in the ER.

Mannose-6-phosphate Tag for Lysosomal Targeting Is Mainly Found on N-84, N-333, and N-393—We finally addressed the function of the individual oligosaccharides of murine ASM in lysosomal targeting. Since each mutant devoid of a single glycosylation site showed significant amounts of intracellular enzymatic activity, the targeting signal is not connected to an individual oligosaccharide. HEK293 cells transiently expressing the mutants ΔGS3 and ΔGS4 secreted considerable ASM enzymatic activity into the cell culture medium. We, therefore, investigated the respective double mutant ΔGS(3+4). No intracellular activity was detectable, but a sig-
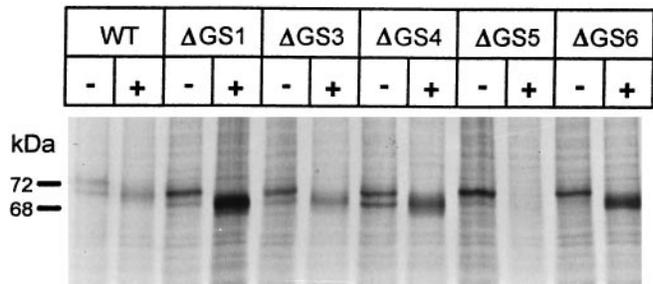
nificant activity was found in the culture medium. The mutant retained a mannose-6-phosphate tag, as demonstrated by immunoprecipitation of ^{32}PO$_4$ pulse-labeled protein (Fig. 9A). The tag remaining in the mutant enzyme is either not sufficient for recognition by the mannose-6-phosphate receptor or the absence of the intracellular activity of this mutant results from a strongly impaired lysosomal protein stability.

To determine the distribution of phosphorylated mannose residues to the individual oligosaccharides, HEK293 cell clones stably expressing wild-type and mutant ASM (WT, ΔGS1 to ΔGS6) were extracted after either [^{35}S]methionine or ^{32}PO$_4$ pulse labeling under comparable conditions. Labeled ASM of each extract was quantified after immunoprecipitation (Fig. 9B). Fig. 9C shows the ratio of ^{32}PO$_4$ to [^{35}S]methionine-labeled ASM for each investigated construct deduced from two independent sets of experiments. Compared to the wild-type, the mutants ΔGS1, ΔGS3, and ΔGS4 exhibited a reduced degree of phosphorylation. This indicated that the respective oligosaccharides were predominant targets for tagging by mannose-6-phosphate. Mutant ΔGS5 also showed an extensively reduced phosphorylation compared to the wild type. However, since this protein is rapidly degraded, insufficient folding and improper molecular structure rather than the elimination of a putatively mannose-6-phosphate tagged oligosaccharide might be the reason for this poor degree of phosphorylation. Therefore, the participation of oligosaccharide at N-518 in mannose-6-phosphate targeting cannot be deduced by this approach.

DISCUSSION

ASM is a heavily glycosylated lysosomal protein. We report here on the various functional aspects of its oligosaccharides. Murine wild-type ASM appears as a 70/72-kDa glycoprotein with five to six *N*-linked oligosaccharides attached. Glycosylation site N-611, which is not conserved between murine and human ASM and therefore should be less important, was found to be only partially used. Elimination of all oligosaccharides led to a 60-kDa polypeptide. Therefore, each oligosaccharide chain contributes an apparent mass of approximately 2 kDa to the molecular mass of the protein. A loss of oligosaccharides during late processing steps cannot be excluded, although not observed during the labeling periods of up to 4 h. Because the cDNA-derived polypeptide without the signal peptide (amino acids 45 to 627) yields a 63-kDa polypeptide, an additional early proteolytic cleavage of an approximately 3-kDa peptide is required to yield the final polypeptide core. In fact, a weak additional protein band of 75 kDa is visible in Fig. 2B (*chase, 0 h*). The absence of this protein band in the other immunoprecipitation experiments might result from the relatively early processing of this product during the long labeling periods. This processing step is in agreement with the observations of Hurwitz *et al.* (27). However, these authors observed an additional 57-kDa early product that did not occur in our experiments.

FIG. 8. **Subcellular localization of wild-type and glycosylation-deficient ASM.** Embryonic fibroblasts of ASM-deficient mice were transfected transiently with ASM expression constructs (*A,* wild type; *B,* ΔGS3; *C,* ΔGS4; *D,* ΔGS5; *E,* ΔGS(3–5)). After 48 h, cells were fixed with a methanol/acetone mixture and labeled with rabbit anti-ASM serum followed by Cy3-conjugated anti-rabbit IgG. The fibroblasts in *F* were labeled with rabbit anti-BiP serum. *Bar,* 20 μm.
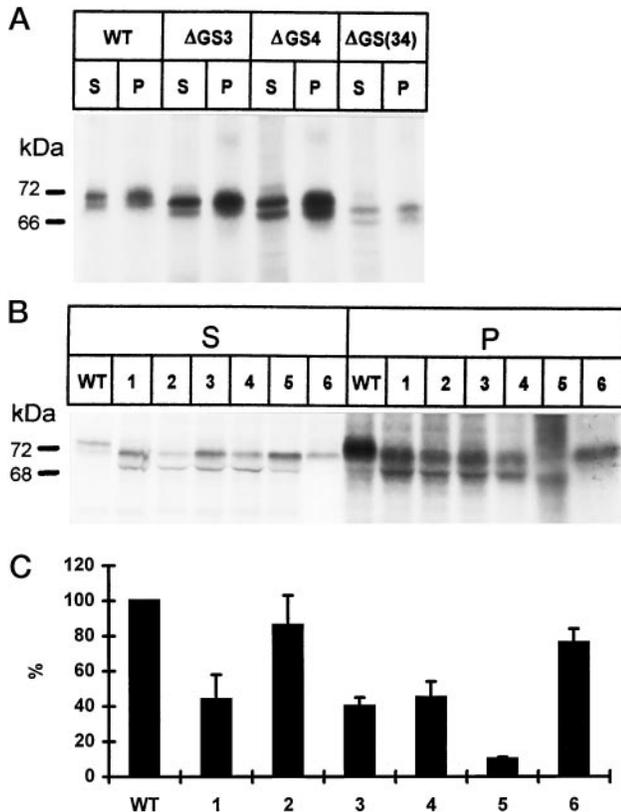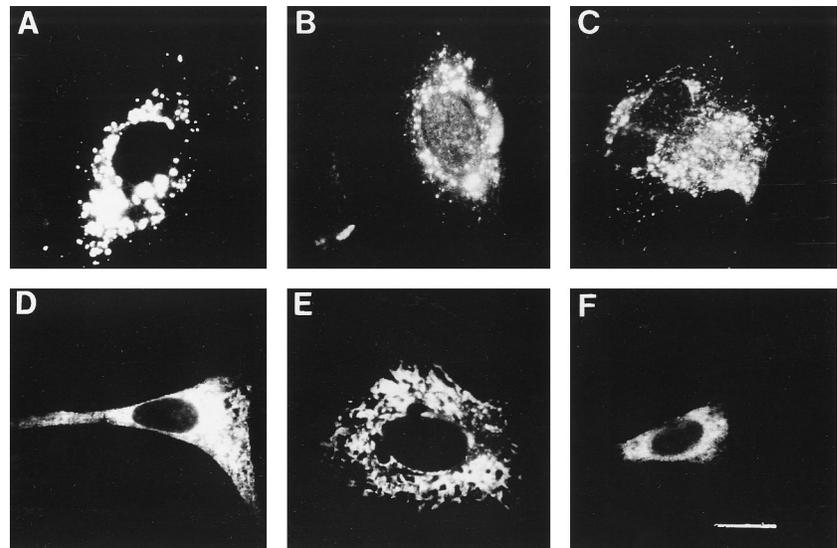

FIG. 9. **Phosphorylation of ASM *N*-linked oligosaccharides.** In *A* and *B,* HEK293 cell clones overexpressing wild-type and mutant ASM were labeled either with [^{35}S]methionine (*S*) or with ^{32}PO$_4$ (*P*) for 3 h, followed by an immunoprecipitation and separation on a 10% SDS-polyacrylamide gel electrophoresis. In *C,* signals of *B* were quantified using a PhosphorImager and the ImageQuant software. The ratio of ^{32}PO$_4$-labeled protein to the respective [^{35}S]methionine-labeled protein was calculated for wild-type (*WT*) and mutant ASM ΔGS1 to ΔGS6 (1–6, respectively).

Variations in the Endo H accessibility of the oligosaccharides observed in this study and by Hurwitz *et al.* (27) might be due to the different cell types chosen for expression. It has been reported that in different tissues different oligosaccharide structures are produced (28).

N-linked oligosaccharides are known to affect the catalytic activity of the glycoproteins in many cases. It has frequently been observed that the *N*-linked oligosaccharides of a protein do not have a profound site-specific effect (24, 29–32). However, the quantitative expression studies of mutated ASM constructs described here revealed only three of the six *N*-linked oligosaccharides (N-333, N-393, and N-518) to be of importance for the enzymatic activity. Similar observations have been reported for human chorionic gonadotropin (33), simian virus 5 hemagglutinin neuraminidase (34), human transferrin receptor (25), and others (35, 36).

Two of the crucial oligosaccharides (N-333 and N-393) were shown to preserve the conformational stability and prevent its proteolytic degradation. The discrepancy between the reduction of immunoprecipitable mutant ASM ΔGS5 (N-518) and its enzymatic activity suggests that a significant amount of enzymatically inactive mutant ASM deficient in this glycosylation site is retained in the ER and rapidly degraded (Fig. 5). This result is also supported by the intracellular distribution of ΔGS5 visualized by immunofluorescence (Fig. 8*D*). We conclude that the oligosaccharide linked at N-518 is essential for the correct folding of the protein.

BiP (grp78) is an ER-located member of the hsp70 family that is known to be involved in glycoprotein folding. Whereas correctly folded and assembled proteins bind only transiently to BiP (20, 21), improper folding leads to stable aggregation. BiP-bound proteins are retained in the ER and degraded rapidly. Several examples of insufficiently glycosylated proteins are reported to be malfolded and, therefore, stably associated with BiP (22–25). We demonstrated by immunoprecipitation that insufficiently glycosylated ASM mutants also coprecipitated with BiP and were retained in the ER as shown by immunofluorescence microscopy. This is particularly obvious for mutant ASM lacking the glycosylation at N-518 (ΔGS5). This suggests that the glycosylation at N-518 prohibits stable binding to BiP. The faint BiP band in Fig. 6 might be due to the relatively long half-life of BiP (37) and the low amount of protein synthesized during the labeling period. Disulfide-linked aggregates as reported for glycosylation-deficient influenza virus hemagglutinin (23), human β-hexosaminidase A (24), or several other proteins (38, 39) did not occur for the ASM (data not shown).

Apart from their influence on protein stability and folding, oligosaccharides of soluble lysosomal glycoproteins are also involved in the correct targeting. Certain oligosaccharides are phosphorylated and subsequently recognized by a Golgi-located mannose-6-phosphate receptor. Although mannose-6-phosphate tagging of cathepsin D has been studied intensively (40, 41), the common features of a recognition domain responsible

for this phosphorylation of lysosomal proteins remains to be determined. Analysis of this phosphorylation is further complicated by the finding that frequently several oligosaccharides of a protein become tagged to a certain extent and, therefore, contribute to the lysosomal targeting. Correspondingly, at least three oligosaccharides of the ASM (N-84, N-333, and N-393) were shown to be phosphorylated to a certain degree. The determination of the distribution of mannose-6-phosphate tags among the oligosaccharides of lysosomal proteins might contribute to a better understanding of this targeting mechanism. Because ASM is highly conserved between humans and mice, it is likely that the respective oligosaccharides of the human ASM have analogous functions.

REFERENCES

1. Quintern, L. E., Weitz, G., Nehrkorn, H., Tager, J. M., Schram, A. W., and Sandhoff, K. (1987) *Biochim. Biophys. Acta* **922,** 323–336
2. Schuchman, E. H., Suchi, M., Takahashi, T., Sandhoff, K., and Desnick, R. J. (1991) *J. Biol. Chem.* **266,** 8531-8539
3. Graber, D., Salvayre, R., and Levade, T. (1994) *J. Neurochem.* **63,** 1060–1068
4. Otterbach, B., and Stoffel, W. (1995) *Cell* **81,** 1053–1061
5. Horinouchi, K., Erlich, S., Perl, D. P., Ferlinz, K., Bisgaier, C. L., Sandhoff, K., Desnick, R. J., Stewart, C. L., and Schuchman, E. H. (1995) *Nat. Genet.* **10,** 288–293
6. Schütze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K., and Krönke, M. (1992) *Cell* **71,** 765–776
7. Kuno, K., Sukegawa, K., Ishikawa, Y., Orii, T., and Matsushima, K. (1994) *Int. Immunol.* **6,** 1269–1272
8. Wiegmann, K., Schütze, S., Machleidt, T., Witte, D., and Krönke, M. (1994) *Cell* **78,** 1005–1015
9. Andrieu, N., Salvayre, R., and Levade, T. (1994) *Biochem. J.* **303,** 341–345
10. von Heijne, G. (1986) *Nucleic Acids Res.* **14,** 4683–4690
11. Halaban, R., Moellmann, G., Tamura, A., Kwon, B. S., Kuklinska, E., Pomerantz, S. H., and Lerner, A. B. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 7241–7245
12. Kretz, K. A., Carson, G. S., Morimoto, S., Kishimoto, Y., Fluharty, A. L., and O'Brien, J. S. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 2541–2544
13. Walsh, M. T., Watzlawick, H., Putnam, F. W., Schmid, K., and Brossmer, R. (1990) *Biochemistry* **29,** 6250–6257
14. Newrzella, D., and Stoffel, W. (1992) *Biol. Chem. Hoppe-Seyler* **373,** 1233–1238
15. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorf, J. W. (1990) *Methods Enzymol.* **185,** 60–89
16. Stoffel, W., LeKim, D., and Tschung, T. S. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352,** 1058–1064
17. Sedmak, J. J., and Grossberg, S. S. (1977) *Anal. Biochem.* **79,** 544–552
18. Duch, M., Paludan, K., Pedersen, L., Jørgensen, P., Kjeldgaard, N. O., and Pedersen, F. S. (1990) *Gene (Amst.)* **95,** 285–288
19. Platt, S. G., and Yang, N. (1987) *Anal. Biochem.* **162,** 529–535
20. Machamer, C. E., Doms, R. W., Bole, D. G., Helenius, A., and Rose, J. K. (1990) *J. Biol. Chem.* **265,** 6879–6883
21. Haas, I. G. (1991) *Curr. Top. Microbiol. Immunol.* **167,** 71–82
22. Dorner, A. J., Bole, D. G., and Kaufman, R. J. (1987) *J. Cell Biol.* **105,** 2665–2674
23. Hurtley, S. M., Bole, D. G., Hoover-Litty, H., Helenius, A., and Copeland, C. S. (1989) *J. Cell Biol.* **108,** 2117–2126
24. Weitz, G., and Proja, R. L. (1992) *J. Biol. Chem.* **267,** 10039–10044
25. Williams, A. M., and Enns, C. A. (1993) *J. Biol. Chem.* **268,** 12780–12786
26. Misumi, Y., Misumi, Y., Miki, A., Takatsuki, A., Tamura, G., and Ikehara, Y. (1986) *J. Biol. Chem.* **261,** 11398–11403
27. Hurwitz, R., Ferlinz, K., Vielhaber, G., Moczall, H., and Sandhoff, K. (1994) *J. Biol. Chem.* **269,** 5440–5445
28. Parekh, R. B., Dwek, R. A., Thomas, J. R., Opdenakker, G., and Rademacher, T. W. (1989) *Biochemistry* **28,** 7644–7662
29. Machamer, C. E., Florkiewicz, R. Z., and Rose, J. K. (1985) *Mol. Cell. Biol.* **5,** 3074–3083
30. Gallagher, P. J., Henneberry, J. M., Sambrook, J. F., and Gething, M. J. (1992) *J. Virol.* **66,** 7136–7145
31. Tifft, C. J., Proia, R. L., and Camerini-Otero, R. D. (1992) *J. Biol. Chem.* **267,** 3268–3273
32. Shipley, J. M., Grubb, J. H., and Sly, W. S. (1993) *J. Biol. Chem.* **268,** 12193–12198
33. Matzuk, M. M., and Boime, I. (1988) *J. Cell Biol.* **106,** 1049–1059
34. Ng, D. T., Hiebert, S. W., and Lamb, R. A. (1990) *Mol. Cell. Biol.* **10,** 1989–2001
35. Dube, S., Fisher, J. W., and Powell, J. S. (1988) *J. Biol. Chem.* **263,** 17516–17521
36. Pique, C., Pham, D., Tursz, T., and Dokhelar, M. C. (1992) *J. Virol.* **66,** 906–913
37. Haas, I. G. (1994) *Experimentia* **50,** 1012–1020
38. Machamer, C. E., and Rose, J. K. (1988) *J. Biol. Chem.* **263,** 5955–5960
39. Marquardt, T., and Helenius, A. (1992) *J. Cell Biol.* **117,** 505–513
40. Baranski, T. J., Faust, P. L., and Kornfeld, S. (1990) *Cell* **63,** 281–291
41. Metcalf, P., and Fusek, M. (1993) *EMBO J.* **12,** 1293–1302