Biochem. J. (1992) 286, 613–618 (Printed in Great Britain)

Human aspartylglucosaminidase

A biochemical and immunocytochemical characterization of the enzyme in normal and aspartylglucosaminuria fibroblasts

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Aspartylglucosaminidase (AGA, EC 3.5.1.26) is an essential enzyme in the degradation of asparagine-linked glycoproteins. In man, deficient activity of this enzyme leads to aspartylglucosaminuria (AGU), a recessively inherited lysosomal storage disease. Here we used affinity-purified polyclonal antibodies against the native AGA and its denatured subunits to establish the molecular structure and intracellular location of the enzyme in normal and AGU fibroblasts. Inactivation of the enzyme was found to coincide with the dissociation of the heterodimeric enzyme complex into subunits. Although the subunits were not linked by covalent forces, the intrapolypeptide disulphide bridges were found to be essential for the normal function of AGA. AGA was localized into lysosomes in control fibroblasts by both immunofluorescence microscopy and immuno-electron microscopy, whereas in AGU cells the location of antigen was different, suggesting that, owing to the mutation, a missing disulphide bridge, most of the enzyme molecules get retarded in the *cis*-Golgi region and most probably face intracellular degradation.

INTRODUCTION

Aspartylglucosaminidase (AGA, EC 3.5.1.26) is a lysosomal amidase that hydrolyses the amide bond between asparagine and *N*-acetylglucosamine in glycoproteins (Makino *et al.*, 1966). The enzyme requires free α-amino and β-carboxy groups of the asparagine residue, and fucose linked to the 6-position of *N*-acetylglucosamine must be removed before cleavage by AGA (Tarentino *et al.*, 1975). We have recently cloned the full-length 2.1 kb cDNA coding for human AGA and established that the enzyme polypeptide is encoded by a single gene in chromosome 4 (Ikonen *et al.*, 1991*a*). On the basis of the sequence data obtained from the cDNA, human AGA is translated as a polypeptide of 346 amino acids containing two potential glycosylation sites. Denaturation of the AGA polypeptide results in 24/25 kDa and 17/18 kDa fragments. The heterogeneity of the molecular masses is caused by heterogeneity in the attached oligosaccharides (Baumann *et al.*, 1989; Fisher *et al.*, 1990; Halila *et al.*, 1991; Ikonen *et al.*, 1991*a*).

The deficiency of this amidase results in the human lysosomal storage disease aspartylglucosaminuria (AGU). The incomplete degradation of glycoproteins leads to accumulation of aspartylglucosamines and other uncleaved glycoasparagines in enlarged lysosomes in all tissues, and secretion of the uncleaved metabolites into the urine (Maury, 1980). The only clustering of AGU patients is found in Finland: more than 200 patients have been found in this genetically isolated population of 5 million, whereas only occasional cases have been reported elsewhere. Our recent studies have shown that a vast majority (98%) of the Finnish AGU patients have the same defect in the gene coding for AGA: two point mutations in the codons coding for amino acids 161 and 163 (Ikonen *et al.*, 1991*a*; Syvänen *et al.*, 1992). With *in vitro* expression studies we recently demonstrated that the latter, a Cys-to-Ser transition (designated the AGU_{Fin} mutation), results in the absence of one intramolecular disulphide bond and is alone sufficient to cause the decreased AGA activity *in vitro* (Ikonen *et al.*, 1991*b*).

Here affinity-purified polyclonal antibodies were raised against the native form of AGA and its denatured 24 kDa and 17 kDa fragments. The antibodies were used to analyse the relationship between 24 kDa and 17 kDa fragments and the native enzyme molecule and for immunocytochemical analyses of AGA both in control and AGU fibroblasts.

MATERIALS AND METHODS

Purification of AGA

Human leucocyte aspartylglucosaminidase was purified from leucocytes originating from 140 litres of blood as described previously (Halila *et al.*, 1991). The purification steps included concavalin A affinity chromatography, Bio-Gel P-100 gel filtration, chromatofocusing with Mono-P f.p.l.c. and reverse-phase h.p.l.c. to separate the subunits of AGA.

Assay of enzyme activity

The AGA activity assay is based on colorimetric measurement of *N*-acetylglucosamine released by the enzyme from the synthetic substrate 2-acetamido-1β-(L-aspartamido)-1,2-dideoxy-β-D-glucose (AADG; Sigma Chemical Co., St Louis, MO, U.S.A.). The assay was carried out using 100 nmol of the substrate AADG in 25 mM-potassium phosphate buffer, pH 6.1, in a final volume of 50 μl, by incubation at 55 °C for 2 h. The reaction was stopped by adding 150 μl of 0.8 M-borate buffer, pH 7.9, followed by heating of the samples at 100 °C for 3 min. The liberated *N*-acetylglucosamine was measured as described by Reissig *et al.* (1955).

To study the effect of SDS on the native conformation of the enzyme, 1.5 μg of 1800-fold-purified enzyme was incubated with

Abbreviations used: AGA, aspartylglucosaminidase; AGU, aspartylglucosaminuria; AADG, 2-acetamido-1β-(L-aspartamido)-1,2-deoxy-β-D-glucose; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate-buffered saline; FCS, foetal-calf serum; PNGase, *N*-glycosidase F; FITC, fluorescein isothiocyanate.

100 nmol of the substrate in the presence of 0, 0.1 or 0.5% SDS at temperatures ranging from 25 to 80 °C.

The effect of 2-mercaptoethanol on AGA activity was measured by incubating 0.8 μg of homogeneous AGA with 0.1–2% 2-mercaptoethanol (Fluka Chemie AG, Buchs, Germany) for 30 min at room temperature before enzyme assay.

Preparation of antiserum against native AGA protein and its isolated subunits

Antibodies against native AGA and its denatured subunits were raised in rabbits. Two rabbits were immunized with Mono-P-purified native AGA enzyme (20 μg/injection). Individual 24 and 17 kDa polypeptides (20 μg/injection), separated by reverse-phase h.p.l.c., were used as antigens for two rabbits each. The rabbits were tested for their antigenic response 1 week after the second booster and bleeded after that (Halila *et al.*, 1991).

Purification of antibodies

The anti-AGA antibodies were purified by affinity chromatography (Tan-Wilson *et al.*, 1976). A 1 mg portion of purified AGA was incubated with CNBr-activated Sepharose 4 B for 2 h at room temperature. The amount of coupled AGA was approx. 170 μg/ml of gel. A 2 ml sample of crude antiserum against native AGA was run through the column at low speed for 16 h at room temperature. After washing the column with 0.15 M-NaCl/0.079 M-sodium phosphate buffer, pH 7.4, the antibodies bound to the enzyme were eluted with 0.2 M-glycine/HCl at pH 2.5. A 100 μl portion of 1 M-Tris was immediately added to the collecting tubes to neutralize the eluting solution. Recovery of the affinity-purified antibody was estimated to be 300 μg by using an $A_{280}^{1\ mg/ml}$ value of 1.38 (Whitaker & Granum, 1980), most of the antibody being in the second fraction.

Immunoprecipitation

Immunoprecipitation was carried out as described by Proia & Neufeld (1981). A 0.3 μg portion of enzyme was incubated with purified IgG (0.5–6 μg) or with antiserum against the native AGA or h.p.l.c.-separated subunits with the corresponding amount of IgG in 0.15 M-NaCl/5 mM-EDTA/50 mM-Tris/HCl, pH 7.4, in a total volume of 50 μl, at 4 °C for 16 h. A 25 μl portion of Protein A–Sepharose suspension was added, and the mixture was shaken for 45 min at room temperature. The immunoprecipitated protein was then collected by centrifugation at 12000 *g* for 5 min. The active enzyme that was not immunoprecipitated was assayed from the supernatant.

Western analysis

For the Western-blot analysis of AGA, SDS (2% final concn.) was added to the samples, and some of them were additionally heated for 3 min at 100 °C before the electrophoresis run on a Phast Gel Gradient (8–25% gel) in a Phast System instrument (Pharmacia, Sweden). The separated proteins were transferred on to nitrocellulose membrane (Hybond-C extra; Amersham International) or poly(vinylidene difluoride) membrane (Immobilon-P; Millipore Co, Bedford, MA, U.S.A.) for 2 h at 70 °C, followed by washing in TBST [10 mM-Tris/HCl (pH 8.0)/150 mM-NaCl/0.05% Tween 20] and blocking in 1% BSA (Sigma) in TBST. The primary antisera were used at 1:300–1:600 dilutions in TBST in the immunostaining, and a 1:7500 dilution of alkaline phosphatase-conjugated anti-rabbit IgG was used as the second antibody using a method provided by the manufacturer (ProtoBlot; Promega, Sweden).

Cells and culture conditions

Skin fibroblasts from an AGU patient (AGU cells) and human embryonic skin fibroblasts (HES cells) were used at their sixth to eight passage. They were grown in Dulbecco's modification of Eagle's medium (DMEM; Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.), supplemented with 2 mM-glutamine, 10% (v/v) fetal-calf serum (FCS) and antibiotics. For immunofluorescence and immunoelectron microscopy, semi-confluent monolayers cultured on glass coverslips were used.

Immunofluorescence microscopy

For immunofluorescence microscopy the cells grown on glass coverslips were fixed in methanol at −20 °C for 10 min and then washed with phosphate-buffered saline (PBS; 145 mM-NaCl/10 mM phosphate buffer, pH 7.4). In order to saturate non-specific protein-binding sites, the cells were incubated with 10% FCS. This was followed by incubation with affinity-purified anti-AGA antibodies at 4 °C for 60 min, and then with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (DAKO, Glostrup, Denmark). The cells were mounted in 0.1–0.2 M-n-propyl gallate solution made in glycerol (Giloh & Sedat, 1982).

In order to stain lysosomes, the unfixed cells were incubated with Acridine Orange (2 μg/ml in Hanks balanced salt solution; Molecular Probes, Eugene, OR, U.S.A.) at 37 °C for 10 min and then mounted in Hanks buffer.

The cells stained for AGA and for lysosomes were viewed under an Olympus BH2 or a Zeiss Axiovert 405 microscope equipped with filters for FITC and tetramethylrhodamine isothiocyanate fluorescence. Kodak TMAX 3200 ASA film was used for photography.

Immunoelectron microscopy

For immunoelectron microscopy the cells were grown in plastic culture dishes as described above. After rinsing with Hanks buffer and PBS, they were gently removed from the bottle with a rubber policeman. The cells were fixed with 8% (v/v) formaldehyde in 0.1 M-phosphate buffer, pH 7.4, for 30 min, immersed in 2.3 M-sucrose, and then frozen in liquid N_2. Thin sections were cut with a Reichert Ultracut FC-E ultramicrotome. Immunolabelling was as follows. The sections were first incubated in 10% FCS containing 0.02% glycine in PBS (PBS/glycine). They were then labelled with affinity-purified anti-AGA antibodies, diluted in 5% FCS in PBS/glycine for 45 min, followed by Protein A–gold complex (particle size 10 nm) for 30 min (Slot & Geuze, 1985). The sections were embedded in methylcellulose and examined in a Philips 410 transmission electron microscope using an acceleration voltage of 60 kV.

Computer analysis

The preliminary analysis of the databases was done by program Pcompare provided by Intelligenetics (Genova, Switzerland). Further, the databases were analysed with the programs FASTA and TFASTA (Lipman & Pearson, 1985). Sequence comparison and comparison of secondary-structural predictions was done as described by Vihinen (1988). Secondary structural elements were predicted by the method of Chou & Fasman (1978), flexibility as described by Karplus & Schulz (1985) and antigenic index by the method of Jameson & Wolf (1988).

RESULTS

Subunit structure and stability of AGA

To identify specifically both the intact AGA polypeptide chain and post-translationally cleaved fragments, we raised antibodies against both the native enzyme and the denatured 24 kDa and 17 kDa fragments. The homogeneous enzymically active human leucocyte AGA was obtained from the last step of the purification procedure, the peak fraction on Mono-P f.p.l.c., and the enzymically inactive 24 kDa and 17 kDa fragments were sepa-

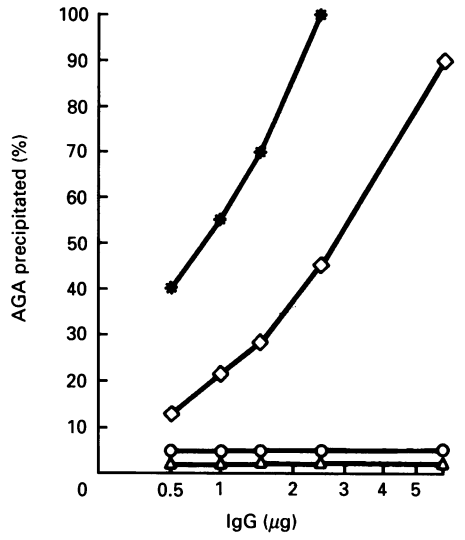

Fig. 1. Immunoprecipitation of AGA activity with anti-AGA antibodies

A 0.3 μg portion of homogeneous AGA was incubated with 0.5–6 μg of anti-AGA IgG in a total volume of 50 μl at +4 °C overnight. The antibody-bound enzyme was immunoprecipitated with Protein A–Sepharose. The unbound enzyme activity was determined from the supernatant as described in the Materials and methods section and expressed as a percentage of the control. □, Antibodies against native AGA; *, affinity-purified antibodies against native AGA; △, antibodies against the 17 kDa subunit; ○, antibodies against the 24 kDa subunit.

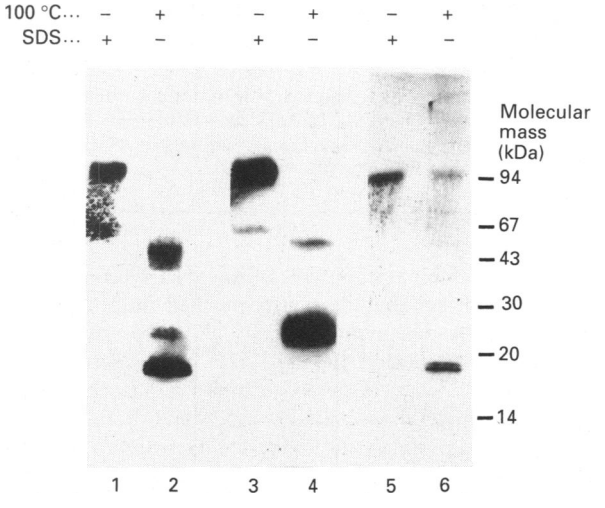

Fig. 2. Western-blot analysis of human AGA

A 0.7 μg portion of protein per lane was run on SDS/8–25% gradient PAGE (Phastgel; Pharmacia) without pretreatment with reducing agent. The proteins were transferred to nitrocellulose at 70 °C and immunostained with antibodies against native AGA (lanes 1 and 2), 24 kDa subunit (lanes 3 and 4) or 17 kDa subunit (lanes 5 and 6). In lanes 1, 3 and 5 only, 0.5% SDS was added to the sample. In lanes 2, 4 and 6 the samples were additionally heated at 100 °C for 3 min. 47 kDa band revealed with native and anti-24 kDa antibodies most probably represents a dimer of two 24 kDa subunits.

rated from the same fraction by reverse-phase h.p.l.c. (Halila *et al.*, 1991). All three antibodies were purified by using the respective antigens coupled to CNBr-activated Sepharose 4B.

Antibodies raised against the homogeneous active AGA identified both native and denaturated forms of AGA enzyme. The enzyme activity could be immunoprecipitated up to 98% (Fig. 1). If the AGA protein was not denatured by boiling with SDS before application on SDS/PAGE in Western-blot analysis, these antibodies identified a high-molecular-mass AGA band representing the native form of the enzyme with detectable enzymic activity (Fig. 2). Boiling of the enzyme with 0.5% SDS before SDS/PAGE and after Western-blot analysis resulted in the disappearance of the high-molecular-mass band and appearance of four bands of molecular mass 17/18 kDa and 24/25 kDa (Fig. 2). Further studies on the effect of SDS on the native AGA enzyme revealed that only temperatures higher than 60 °C resulted in the decrease of AGA activity in the presence of 0.1–0.5% SDS at pH 7 (Fig. 3), demonstrating the AGA protein's exceptional resistance to SDS at lower temperatures.

The antibodies raised against 24 kDa and 17 kDa fragments separated by h.p.l.c. detected only the corresponding AGA

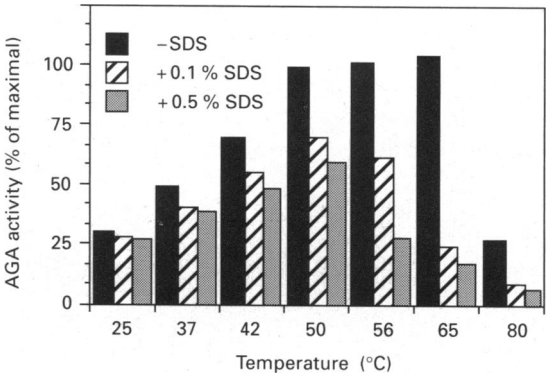

Fig. 3. Effect of SDS on AGA activity at different temperatures

Partially purified AGA protein was incubated with AADG under the conditions described in the Materials and methods section at temperatures from 20 to 80 °C in the presence of 0, 0.1 or 0.5% SDS. Activities are expressed as percentage of maximal activity. Black bars show AGA activity without SDS, hatched bars show AGA activity in the presence of 0.1% SDS, and stippled bars show AGA activity in the presence of 0.5% SDS.

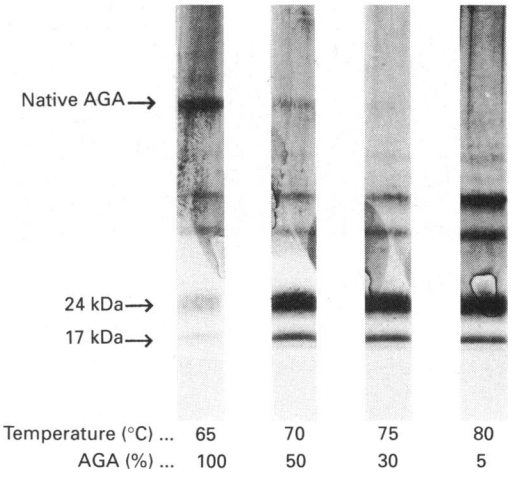

Fig. 4. Temperature-dependent separation of subunits

A 0.3 μg sample of AGA was incubated at temperatures ranging from 65 to 80 °C for 10 min and then directly applied to an SDS/gradient 8–25% PAGE gel (Phastgel, Pharmacia). The proteins were transferred to PVDF membrane at 70 °C. Immunostaining of the lanes was carried out using subunit-specific antibodies as a mixture in a dilution of 1:200. The two invariant bands about 47 kDa and 36 kDa most probably represent dimers of 24 kDa and 17 kDa subunits respectively.

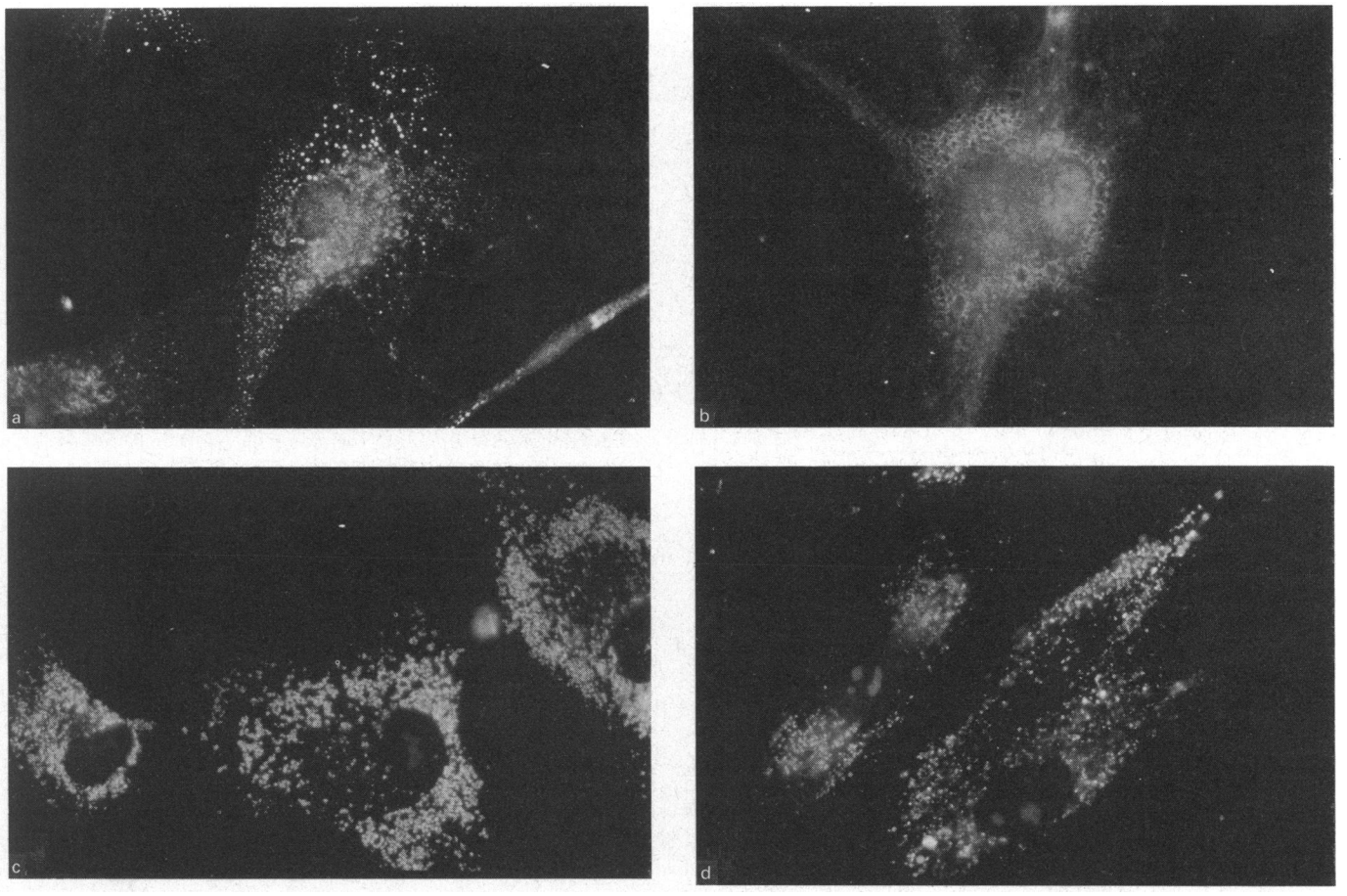

Fig. 5. Immunofluorescence micrographs of HES cells (a and b) and AGU cells (c and d)

a and c show immunolabelling for AGA with anti-AGA antibodies, and b and d show vital staining for lysosomes with Acridine Orange. In HES cells a punctate staining pattern can be seen with both anti-AGA antibodies and Acridine Orange (a and b). In AGU cells, anti-AGA antibodies give a lace-like staining pattern (c) that is different from anti-AGA staining in HES cells (a) and from Acridine Orange staining in AGU cells (d). Magnification × 180.

polypeptides in Western analysis when the sample was boiled before application on to the gel (Fig. 2, lanes 4 and 6). In this analysis the subunit-specific antibodies did not cross-react with each other. They detected neither the native form of AGA when Western analysis was carried out after PAGE without SDS nor immunoprecipitate AGA activity in solution (Fig. 1). However, when the electrophoresis was carried out under non-denaturing conditions and the polypeptide chains were heat-denatured only during transfer to the membrane, these antibodies detected the same high-molecular-mass band as the antibodies against native AGA (see above) (Fig. 2, lanes 3 and 5).

The relationship between denaturation of the enzyme and dissociation into subunits was studied further by Western Blotting after incubation of the enzyme at different temperatures. The high-molecular-mass band corresponding to the native form of AGA disappeared gradually at higher temperatures and was totally abolished after treatment at 75 °C. The quantity of detectable native form of AGA polypeptide reflected the assayed enzyme activity, although in Western-blot analyses the quantification cannot be precise (Fig. 4). The subunits of AGA are not held together by disulphide bridges, since their appearance did not require pretreatment with mercaptoethanol (Fig. 2). However, the enzyme activity was found to be significantly decreased in the presence of mercaptoethanol. The tested concentrations varied from 0.1 to 2%, and as little as 0.1% resulted in 90% inhibition of AGA activity.

During the preliminary analyses of the glycosylation of AGA we originally observed that the affinity-purified antibodies against the native AGA surprisingly identified also bacterial *N*-glycosidase F (PNGase; EC 3.5.1.52) from *Flavobacterium meningosepticum*. Since both AGA and PNGase are able to hydrolyse the *N*-acetylglucosamine–asparagine bond, the similarity of these two proteins was first tested by program Pcompare, which compares the sequences, calculates their similarity and displays their alignment by using the method of Needleman & Wunsch (1970). An alignment score of 8.3 was found (a score above 5.0 is required for significant alignment if the two sequences have no known relation). Further comparisons of amino acid sequences revealed only 35.3% similarity and 13.1% identity throughout the complete sequences. However, when comparing the predicted secondary-structural elements and the predicted antigenic properties of the proteins by using program package MULTICOMP (Vihinen, 1988; Vihinen *et al.*, 1992), the proteins were found to share four regions of secondary-structural similarity and two additional regions which shared similar antigenic characters between AGA and PNGase (from positions 113 to 130 and from positions 291 to 331).

Intracellular localization of AGA

Localization of AGA by immunofluorescence in normal control fibroblasts revealed a punctate staining pattern. The staining was more pronounced in the perinuclear region, but could also

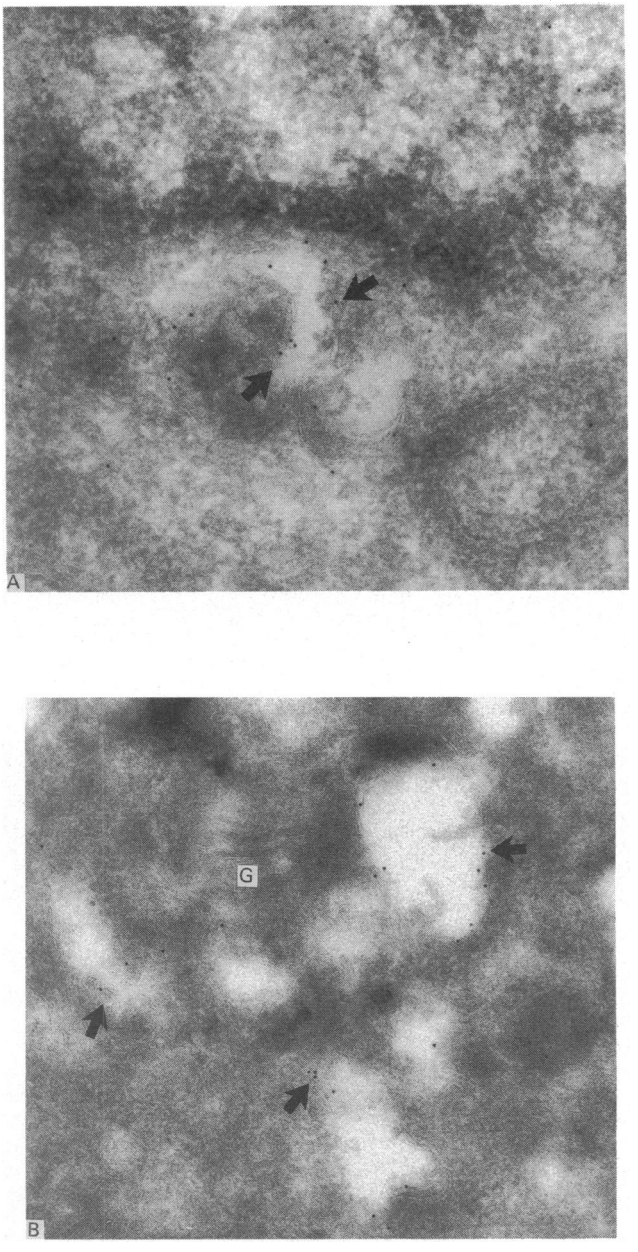

Fig. 6. Immunogold-labelling, with anti-AGA antibodies, of human embryo skin fibrobroblasts (A) and AGU cells (B)

A, gold particles are located along the membrane of vacuoles (arrowheads) resembling lysosomes. B, gold particles are seen along the membrane of large vesicles that constantly lie close to Golgi apparatus (G). Magnification in both A and B, ×40000.

be seen in the peripheral parts of the cells (Fig. 5). A similar staining pattern could be seen in cells stained with Acridine Orange, indicating that the granules positive for AGA represent lysosomes. Immunoelectron microscopy of normal fibroblasts also showed anti-AGA reactivity in association with lysosomes; the individual gold particles could often be seen close to the bilayered membrane enshielding lysosomes (Fig. 6).

In AGU cells, immunofluorescence microscopy with affinity-purified anti-AGA antibodies gave distinctly different staining from that seen in control cells; instead of a punctate staining pattern, there was a more homogeneous perinuclear staining with a lace-like pattern. This was also different from the staining pattern seen in AGU cells that had reacted with Acridine Orange, which showed a granular staining similar to that seen with Acridine Orange in control cells (Fig. 5). In immunoelectron microscopy most of the label was seen in membrane-bound vacuoles, which were frequently found close to the Golgi apparatus. They appeared clearly separate from the convoluted cisternae of the endoplasmic reticulum and ordered stacks of the *trans*-Golgi, and most probably represent widened cisternae of the *cis*-Golgi (Fig. 6).

DISCUSSION

The recent isolation of the AGA cDNA has revealed the complete primary structure of the AGA protein (Fisher *et al.*, 1990; Ikonen *et al.*, 1991*a*), but the subunit structure of the enzyme molecule has remained undefined, and currently very little is known about the intracellular processing of AGA. Human AGA was considered to be a monomer of 80 kDa by McGovern *et al.* (1983), whereas Braumann *et al.* (1989) reported more complex structure of AGA, with three non-identical polypeptides and a native molecular mass of approx. 60 kDa. The homogeneous AGA purified from isolated leucocytes was found to contain two non-identical fragments, which are heterogeneous in their glycosylation (Halila *et al.*, 1991). The term 'subunit' is here used for these two fragments.

Post-translational modifications are typical for lysosomal proteins, which are generally synthesized as high-molecular-mass precursors and further cleaved to multisubunit structures. The proteolytic processing of soluble lysosomal proteins requires acidic pH and often occurs after their transport to the lysosomes. In the case of several multimeric lysosomal hydrolases the subunits in a heteromultimer are encoded by several genes (Proia *et al.*, 1984; Verheijen *et al.*, 1985). AGA is another type of heterodimer, generated by a post-translational proteolytic cleavage of one precursor chain. The predicted cleavage site between the subunits is a stretch of hydrophilic residues upstream of the *N*-terminus of the 17 kDa subunit, a region susceptible to proteolytic hydrolysis on the basis of the deduced amino acid sequence (Fisher *et al.*, 1990; Ikonen *et al.*, 1991*a*). We demonstrate here that both the 24 kDa and 17 kDa subunits are found in the homogeneous active enzyme and that only a combination of both subunits, held together by non-covalent forces, can produce an active enzyme. This is in agreement with our earlier finding that separation of the subunits by reverse-phase chromatography constantly inactivated the enzyme (Baumann *et al.*, 1989; Halila *et al.*, 1991). Findings on human AGA are also supported by the data reported from the purified rat enzyme by Tollersrud & Aronson (1989).

On the basis of the molecular mass obtained from gel-filtration analysis of the purified human enzyme (Halila *et al.*, 1991), the most probable structure of AGA would be a dimer of 24 kDa and 17 kDa subunits. Silver staining of the purified AGA polypeptide chain after SDS/PAGE suggests that the stoichiometric ratio of these subunits is 1:1 (Halila *et al.*, 1991). The results of semi-quantitative Western analyses presented here also support this ratio. So far we have no data of the suggested tetramer structure of AGA (Kaartinen *et al.*, 1991). It remains to be established whether the AGA enzyme is catalytically active in its single-chain, uncleaved, form or whether the activity is generated by the proteolytic cleavage and proper spatial orientation of subunits.

The mature AGA protein contains nine cysteine residues on the basis of the amino acid sequence deduced from the cDNA. We found that although interchain S–S bonds do not hold the 24 kDa and 17 kDa subunits together, intrachain disulphide bridges seem to be essential for the enzyme function. The enzyme activity is decreased after treatment with reducing agents and the

AGU_{Fin} mutation abolishing one disulphide bridge results in deficient enzyme activity (Ikonen *et al.*, 1991*b*). Further, since disulphide linkages between cysteine residues are known to be effective stabilizers of the secondary structure of proteins (Kauzmann, 1959; Pakula & Sauer, 1989), the high number of cysteine residues in AGA could explain its high thermostability (Baumann *et al.*, 1989; Tollersrud & Aronson, 1989) of this enzyme as well as its relative resistance to SDS observed here.

Aspartylglucosaminidase has been classified as a lysosomal amidase, largely because of the lysosomal accumulation of non-cleaved glycoasparagine in AGU patients. However, the lysosomal location of AGA has not been shown unequivocally. Mahadevan & Tappel (1967) reported the presence of AGA activity in rat liver lysosomes by relatively crude subcellular fractionation. This technique is not completely satisfactory, since cross-contamination of isolated fractions can rarely be definitely excluded. In the present study the precise location of AGA was determined by immunocytochemical and immuno-EM techniques. AGA was localized in lysosomal vesicles of cultured human skin fibroblasts, and a strikingly different location of AGA polypeptides was found in AGU cells. The dense perinuclear staining seen in AGU cells is more indicative of AGA located in non-lysosomal than in lysosomal organelles. By its immunoelectron microscopic features this compartment was found to be closely associated with the Golgi apparatus and most probably represents altered *cis*-Golgi cisternae. Also, our previous '*in vitro*' expression studies in COS cells suggest that only a minority of AGA polypeptide chains are exposed to lysosomal proteolysis in fibroblasts expressing the AGU_{Fin} mutation (Ikonen *et al.*, 1991*b*).

It has become apparent that the correct folding is necessary for efficient intracellular transport of proteins, presumably because unfolded proteins tend to bind to other proteins in the endoplasmic reticulum and face intracellular degradation (Pelham, 1989); alternatively, some proteins are transported out of the endoplasmic reticulum before degradation (Le *et al.*, 1990). Mutagenesis studies *in vitro* have actually demonstrated that formation of intramolecular disulphide bonds is a key step in acquiring the completely folded three-dimensional structure of some newly synthesized proteins (Wendland *et al.*, 1991). The AGU mutation analysed here provides direct evidence that intrapolypeptide disulphide bonds are essential for normal folding and intracellular transport of AGA. The Cys-to-Ser mutation, causing the loss of one intrachain disulphide bridge of the enzyme molecule, is clearly followed by misfolding and false compartmentalization of the mutated AGU protein, which eventually results in a severe inherited human disease.

This work was supported by grants from the Academy of Finland, the Rinnekoti Foundation, the Sigrid Juselius Foundation, the Orion Foundation and the Farmos Foundation. We thank Ms. Helena Rantanen for secretarial help, Dr. Ole-Kristian Tollersrud for help and advice in protein analyses, and Dr. Veli-Pekka Lehto for supervising the electron microscopy.

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Received 25 November 1991/14 February 1992; accepted 26 February 1992