Clinical Diversity in Glycogenosis Type II

Biosynthesis and In Situ Localization of Acid α-Glucosidase in Mutant Fibroblasts

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Abstract

The molecular basis of clinical diversity in glycogenosis type II (Pompe's disease) was investigated by comparing the nature of acid α-glucosidase deficiency in cultured fibroblasts from 30 patients. Biosynthetic forms of acid α-glucosidase with different molecular mass were separated electrophoretically and identified by immunoblotting. Immuno–electron microscopy was employed to determine the intracellular localization of mutant enzyme. Our studies illustrate that maturation of acid α-glucosidase is associated with transport to the lysosomes. Deficiency of catalytically active mature enzyme in lysosomes is common to all clinical phenotypes but, in the majority of cases, is more profound in early onset than in late onset forms of the disease. Thus, the results suggest that the clinical course of glycogenosis type II is primarily determined by the amount of functional acid α-glucosidase. The role of secondary factors can, however, not be excluded because three adult patients were identified with very low activity and little enzyme in the lysosomes.

Introduction

Glycogenosis type II is an autosomal recessive disorder characterized by lysosomal accumulation of glycogen and deficiency of acid α-glucosidase (EC 3.2.1.20) (1). Cardiomegaly, hepatomegaly, and severe hypotonia are typical symptoms of the infantile form of the disease, which is rapidly progressive and fatal in the first or second year of life (2, 3). When glycogenosis type II presents at a more advanced age, the symptoms are generally limited to weakness of the skeletal muscles. Patients with these milder forms of the disease have been classified as juvenile or adult variants (3–6).

Some studies on skeletal muscle biopsy material (7), leukocytes (8, 9), cultured skin fibroblasts (10, 11), and urine (12) from patients with glycogenosis type II have suggested that the residual activity of acid α-glucosidase is an important parameter in determining the clinical phenotype. Other studies, however, in which very low enzyme activities were measured in cultured skin fibroblasts and muscle tissue from adult patients, dispute the correlation between the acid α-glucosidase activity and the expression of the disease (13–16). Particular attention has been paid to a possible role of neutral maltases in influencing the pathogenesis of glycogenosis type II, but the results have been contradictory and inconclusive (15–18).

To evaluate the contribution of secondary factors, it is essential to determine at first to what extent clinical diversity is caused by heterogeneity of the acid α-glucosidase locus itself. Indicative of genetic heterogeneity are differences in the acid α-glucosidase activity and the amount of immunologically detectable enzyme protein among clinical variants (10, 11, 13, 16, 19). More recent studies on the biosynthesis of acid α-glucosidase in mutant fibroblasts have given additional and more detailed information on the occurrence of a variety of molecular defects that can lead to glycogenosis type II (14, 20, 21). However, the number of patients in these studies has been too small to investigate properly the relation between specific molecular defects and clinical variation. This question is the subject of the present investigation, in which 30 cases of glycogenosis type II were surveyed. Immunoblotting was used to characterize and quantitate the biosynthetic forms of acid α-glucosidase in the patient's cultured fibroblasts, and the enzymatic activity was determined. Immuno–electron microscopy was employed to study the in situ localization of the mutant enzymes.

Novel defects in the biosynthesis of acid α-glucosidase were discovered. By combining assessment of clinical phenotype, intracellular localization of acid α-glucosidase, and enzymatic activity, it is concluded that the amount of functional enzyme in the lysosome is the primary but probably not the exclusive determinant of the clinical course of glycogenosis type II.

Methods

Cell culture. Fibroblasts from healthy individuals and patients with a clinical and biochemical diagnosis of glycogenosis type II were cultured in Dulbecco's modified Eagle's medium (Flow Laboratories, Inc., McLean, VA), supplemented with 10% fetal calf serum (FCS) (Flow Laboratories, Inc.) and antibiotics. References to the origin of the cell lines are given in Table I.

Biochemical assays and immunoblotting. 3-d confluent cultures were harvested with trypsin and pellets of 3×10^6 fibroblasts were homogenized by sonication in 200 μl of 10 mM sodium phosphate buffer, pH 6.5, containing 0.5 M NaCl. Cell debris was removed by centrifugation for 15 min at 10,000 *g* and the protein concentration was measured by the method of Lowry et al. (22). Lysosomal enzyme activities were determined with the appropriate 4-methylumbelliferyl substrates as described by Galjaard (23). The activity of acid α-glucosidase towards the natural substrate glycogen was measured by incubating 10 μl cell homogenate for 30 min at 37°C with 50 μl substrate solution containing 50 mg glycogen (Gibco, Grand Island, NY) per ml potassium phosphate (0.2 M)–sodiumcitrate (0.1 M), pH 4.4. The reaction was terminated by heating the samples for 1 min at 95°C, and the amount of liberated glucose was determined as described before (10).

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Table I. Origin of Cell Lines and Molecular Features of Acid α-Glucosidase

Code	Clinical subtype	Reference*	α-Glucosidase activity‡ MU	α-Glucosidase activity‡ Glucose	Present age or age at death	Immunoblotting pattern§	Remarks
					yr		
81RD238	Healthy	14	155.5	1086.1	5	control	
JJ	Healthy		127.8	929.2	35		
TRI	Healthy		167.3	1395.3	39		
371LAD	Infantile		0.9	21.1	p.d.	371LAD	sibs
79RD34	Infantile		0.5	9.8	p.d.		
266LAD	Infantile	14	0.8	12.0	0.83‖		
80RD156	Infantile		0.4	30.9	1‖		
131LAD	Infantile		0.9	21.1	1‖		
217LAD	Infantile	14	0.5	19.2	0.5‖		
81RD27	Infantile		0.6	4.6	0.5‖		
658LAD	Infantile		0.3	44.4	1‖	372LAD	
372LAD	Infantile		1.0	15.8	3‖		
460LAD	Infantile		1.4	47.3	0.67‖		
75RD100	Infantile		0.7	36.1	1.5‖		
124LAD	Juvenile	14	2.3	14.5	18‖	82RD101	
82RD101	Juvenile		7.6	61.7	7‖		
77RD84	Juvenile	14	8.1	45.3	20	77RD84	
GM1935	Adult	13, 14	1.2	17.9	35	GM1935	
E.M.	Adult	27	1.3	20.4	35		
84RD390	Adult		4.7	71.9	43		
82RD167	Adult	14	32.5	329.7	72‖		grandfather of 266LAD
79RD102	Adult		34.1	220.9	53	79RD102	
196LAD	Adult	14	14.7	91.8	36		
80RD238	Adult		18.3	188.1	59		
80RD158	Adult		22.8	125.7	45		
79RD196	Adult		30.0	142.3	33		
338LAD	Adult		39.5	174.5	55‖		
356LAD	Adult		29.4	214.0	55		
M.R.	Adult	27	31.2	ND	43		sibs
R.B.	Adult	27	16.7	ND	50		
J.L.	Adult		22.0	114.8	34		
J.S.	Adult	27	28.8	ND	42		
A.S.	Adult	27	34.4	136.7	43	A.S.	

* The cell lines without reference number were obtained from Dr. M. F. Niermeijer, Department of Clinical Genetics, University Hospital, Rotterdam. J.L. was obtained from St. Thomas' Hospital, London. Cell line GM1935 was obtained from the Human Mutant Cell Repository, Institute for Medical Research, Camden, NJ. ‡ Measured in triplicate and expressed in nanomoles methylumbelliferone (MU) or nanomoles glucose per hour per milligram protein. ND, not determined. § Molecular species of acid α-glucosidase in each individual were studied by immunoblotting. Cell lines with same "immunoblotting pattern" are grouped. Cell line representative for each group, illustrated in Fig. 3, is indicated. Immunoblotting patterns of 371LAD, 82RD101, and GM1935 are indistinguishable. ‖ Indicates age at death, otherwise present age is indicated. p.d., prenatal diagnosis confirmed after abortion.

For immunoblotting, 200 μl of a 1:1 suspension of Concanavalin A/Sepharose 4B in homogenization buffer was added to 75 μl of cell extract to bind acid α-glucosidase and other glycoproteins. The binding was carried out overnight at 4°C in a rotating tube. The Sepharose beads were collected by centrifugation and washed five times with homogenization buffer to remove nonspecifically bound proteins. The beads were finally resuspended in 100 μl sample buffer (100 mM Tris-HCl, pH 6.8, 4% (wt/vol) sodium dodecyl sulfate (SDS), 0.6 M mercaptoethanol, 2 M glycerol), and the samples were heated at 90°C for 4 min. The solubilized proteins were separated in 10% polyacrylamide gels containing SDS according to Laemmli (24) and subsequently transferred to nitrocellulose filters as described by Towbin et al. (25).

Polyclonal rabbit anti-human placental acid α-glucosidase antibodies were used for the detection of acid α-glucosidase (14). Immune complexes were visualized autoradiographically via binding of ^{125}I-labeled protein A (25). Antibodies reacting with the 76-kD molecular form of acid α-glucosidase were prepared by application of the method of Smith and Fisher (26). Briefly, the 95, 76, and 70-kD forms of placental acid α-

glucosidase were separated in an 8% SDS-polyacrylamide gel and transferred to nitrocellulose. The bands were cut out separately and used to adsorb immunoglobulins from the polyclonal antiserum. Immunoglobulins were then eluted from the nitrocellulose strips with 5 mM glycine-HCl, pH 2.3, and the pH was readjusted to 7.6.

Immunoprecipitation. Confluent fibroblasts were cultured for 4 h in the presence of L-(4,5-^{3}H)leucine (0.2 mCi; 135 Ci/mmol; Amersham Radiochemical Center, Amersham International, Amersham, UK) and either harvested immediately or cultured for another 4 h in the absence of label before harvesting. Homogenates were prepared and acid α-glucosidase was analyzed in SDS-polyacrylamide gels after immunoprecipitation as described by Hasilik and Neufeld (28, 29).

Immunocytochemistry. 3-d confluent cultures (3–4 × 10^6 cells in total) were harvested with 0.25% trypsin in phosphate buffered saline (PBS), pH 7.0. The period of trypsinization, at 37°C was < 1 min, and detachment of cells was promoted by shaking. The action of trypsin was terminated by resuspending the cells in fresh culture medium containing 10% FCS. Cells were collected by centrifugation, washed two times with fresh medium, and then resuspended in 25 ml of culture medium. They were left for 2 h at 37°C in a rotating tube to recover from possible trypsinization damage. Finally, the cells were pelleted and prepared for immunocytochemistry as described before (30).

Ultrathin frozen sections were incubated with affinity-purified antibodies against placental acid α-glucosidase (14), and antibody-antigen complexes were visualized by a second incubation with goat anti-rabbit immunoglobulins coupled to colloidal gold.

Results

The patients from whom cell lines were obtained were classified as infantile, juvenile, or adult variants of glycogenosis type II according to age of onset, progression, and severity of symptoms (Table I). By far the lowest residual activities of acid α-glucosidase were measured in fibroblasts from patients with severe, early-onset forms of the disease. The enzymatic activity in fibroblasts from most adult patients was found to be substantially higher, with the exception of cell lines GM1935, 84RD390, and E.M. In juvenile forms of glycogenosis type II, the activities were between the infantile and adult ranges. Essentially the same results were obtained with the artificial 4-methylumbelliferyl-α-glucoside substrate as with the natural substrate glycogen (Table I). The activities of two other lysosomal enzymes, β-galactosidase and β-hexosaminidase, were normal in all mutant cell lines (not shown).

The biosynthesis of acid α-glucosidase was studied in several of these mutant cell lines by pulse-chase labeling. For this purpose, the fibroblasts were grown in medium containing [^{3}H]leucine, and acid α-glucosidase was immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis. Fig. 1 shows that in control cells acid α-glucosidase is synthesized as a precursor with an apparent molecular mass of 110 kD and is processed via a 95-kD intermediate to a major species of 76 kD. These conversions are completed within ~ 8 h (14). The faint bands with an apparent molecular weight of < 76 kD are contaminants that also appear when antibodies against β-hexosaminidase are employed. Fig. 1 also illustrates three examples of abnormal enzyme formation. In one patient with the infantile form of glycogenosis type II (266LAD) no acid α-glucosidase polypeptides were detectable. In contrast, the formation of the 110-kD precursor appeared undisturbed in two other mutants (460LAD, infantile, and 84RD390, adult), but here posttranslational processing to mature enzyme was impaired. The 95-kD intermediate form of acid α-glucosidase was present in cell line 460LAD but undetectable in cell line 84RD390 (Fig. 1).

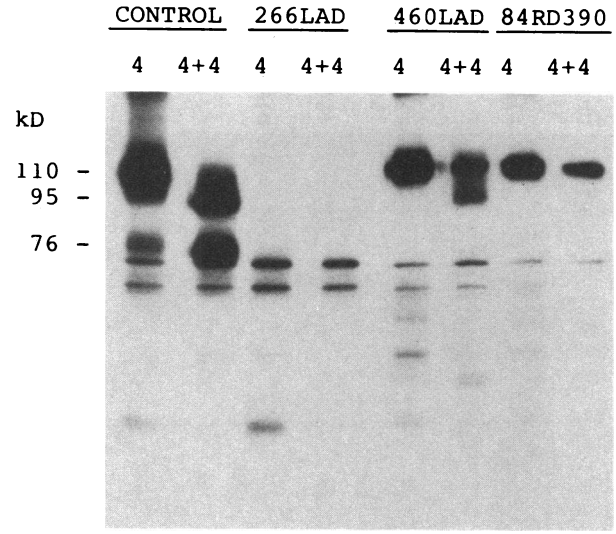

Figure 1. In vitro labeling studies of biosynthetic forms of acid α-glucosidase in normal and mutant fibroblasts. Acid α-glucosidase was biosynthetically labeled with [^{3}H]leucine and immunoprecipitated with affinity-purified antibodies in the presence of protein A coupled to Sepharose-4B. The molecular forms of acid α-glucosidase were separated by SDS–polyacrylamide gel electrophoresis and visualized autoradiographically. The synthesis and processing of acid α-glucosidase was followed by labeling the cells for 4 h, whereafter one lot of cells was harvested (pulse) and a second lot was maintained in fresh medium without [^{3}H]leucine for another 4 h (chase). Clinical subtypes: 266LAD, infantile; 460LAD, infantile; 84RD390, adult.

Immunoblotting was used as a second alternative method to study the molecular forms of acid α-glucosidase in mutant cells. Fig. 2 illustrates the results obtained when this method was applied to the same cell lines as used in Fig. 1. Preparations of acid α-glucosidase purified from human placenta and human urine served as reference samples. Human urine is enriched in precursor acid α-glucosidase and gives a strong signal at the 110 kD position (Fig. 2) (31). Human placenta, on the other hand, contains mainly two mature forms of acid α-glucosidase with an apparent molecular mass of 76 kD and 70 kD (Fig. 2). The molecular pattern of acid α-glucosidase as revealed by immunoblotting of control fibroblasts is similar to the one obtained via biosynthetic labeling except that the 76-kD band is relatively stronger and an additional molecular species of 70 kD is seen. The patterns in the mutant cell lines are also comparable using the two methods, but there are obvious differences. In the cell line 460LAD the 76-kD form of acid α-glucosidase is clearly visible by immunoblotting, and cross-reactive material is detectable in mutant 266LAD at the position of the 110-kD precursor. This band in cell line 266LAD was further identified as the precursor of acid α-glucosidase by its positive reaction with antibodies directed against the 76-kD mature form of the enzyme (Fig. 2).

The immunoblotting procedure was subsequently employed for the screening of a large number of mutant cell lines. The results are presented in Fig. 3 and Table I. Two distinct abnormalities in the formation of acid α-glucosidase were detected among 11 patients with an infantile form of the disease. In seven cases, the 110-kD precursor of acid α-glucosidase was the only detectable band on immunoblots, as illustrated for 371LAD. Processed forms of the enzyme were visible in the remaining

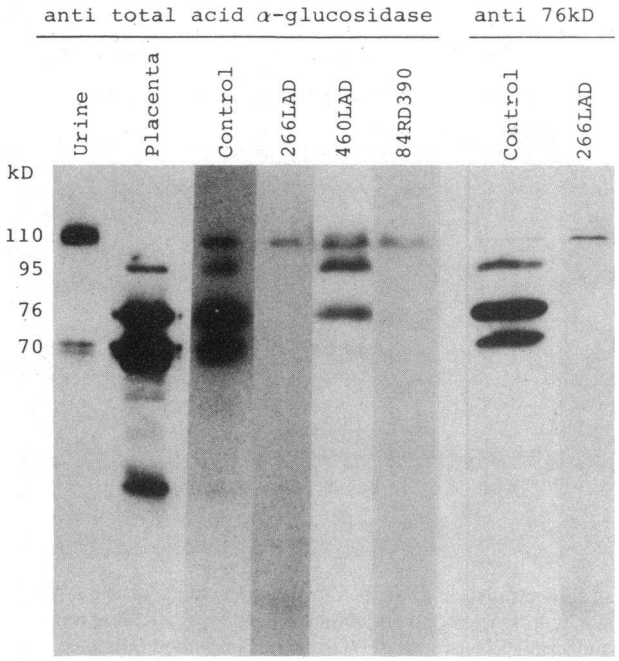

Figure 2. Immunoblotting studies of biosynthetic forms of acid α-glucosidase in normal and mutant fibroblasts. Fibroblast extracts were prepared and acid α-glucosidase was partially purified through binding to concanavalin A–Sepharose 4B. The glycoproteins bound to the beads were dissolved in "sample buffer" and separated by SDS–polyacrylamide gel electrophoresis. After separation they were electrophoretically transferred to nitrocellulose, and acid α-glucosidase was visualized by incubating the filters with antibodies against acid α-glucosidase or with affinity-purified antibodies against the 76-kD mature form of acid α-glucosidase. The immune complexes were detected by a second incubation with ^{125}I-protein A followed by autoradiography. Purified mature α-glucosidase from placenta and precursor enzyme from human urine were applied directly to the gel.

four cases, as illustrated for 372LAD, but these were present in markedly reduced quantities compared with those detected in control fibroblasts.

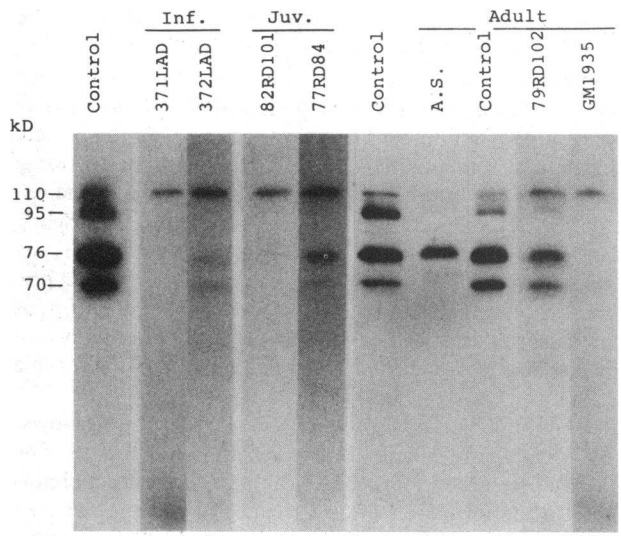

Figure 3. Immunoblotting studies of biosynthetic forms of acid α-glucosidase in mutant fibroblasts; For methods, see legend to Fig. 2. *Inf.*, infantile; *Juv.*, juvenile.

Two types of molecular defect were discovered among three juvenile cases of glycogenosis type II. The precursor was detectable in fibroblasts from all three patients, but conversion to mature enzyme was only evident in the cell line 77RD84. However, the amount of mature enzyme was reduced in comparison with normal.

Also in 13 out of 16 cell lines from adult patients, as illustrated for 79RD102, both the 76 and 70-kD mature forms of acid α-glucosidase were present, in higher amounts than in infantile cases but lower than in healthy individuals. Among these 13 adult cases, variant A.S. was exceptional because some of the molecular components had a slightly different electrophoretic mobility compared with those of other adult and control cell lines. Surprisingly, the three other adult variants showed a molecular pattern very similar to that seen in the majority of infantile cases, with only the 110-kD precursor detectable, as illustrated for GM1935 (Fig. 3). The acid α-glucosidase activity of these three cell lines was profoundly deficient, not only with the artificial substrate but also with the natural substrate glycogen. The results are summarized in Table I.

Considering that the maturation of acid α-glucosidase encompasses several proteolytic cleavage steps and carbohydrate side-chain modifications, one can envisage that several mutations in the enzyme may hamper essential modification events and indirectly obstruct transport to the lysosomes. This would further impair functioning of the mutant enzyme. With this in mind we have studied the intracellular localization of acid α-glucosidase in fibroblasts from eight patients with a different type of processing defect. To visualize acid α-glucosidase in electron micrographs, ultrathin frozen sections of fibroblasts were incubated with specific antibodies and the immune complexes were traced with an electron-dense complex of goat anti–rabbit IgG coupled to colloidal gold.

In the control cells the largest number of gold particles was found in the lysosomes (Fig. 4). In addition, gold particles were detected in or near the cisternae of the rough endoplasmic reticulum (RER).[1] This intracellular compartment is recognized by the presence of limiting membranes that appear white on the picture and darker ribosomes surrounding these membranes (Fig. 4 *A*). Furthermore, acid α-glucosidase was localized in the Golgi complex that was weakly labeled (Fig. 4 *B*). Virtually no background labeling was observed with antibodies against human albumin, thus indicating the specificity of the labeling procedure.

Semiquantitative data on the lysosomal labeling were obtained by counting the number of gold particles in 100 randomly chosen lysosomes (Fig. 5). On the average, 11.5 gold particles were present in lysosomes of control cell line 81RD238. With anti–albumin antibodies, this number was 0.03.

In Fig. 6, the localization of acid α-glucosidase is compared in fibroblasts from three patients with infantile forms of the disease. In the cell line 266LAD (residual activity 0.5%), in which the 110-kD precursor was only detectable via immunoblotting (Fig. 2) but not via immunoprecipitation (Fig. 1), acid α-glucosidase was predominantly found in the RER (Fig. 6 *A*) and in the Golgi complex (Fig. 6 *B*). The intracellular localization of acid α-glucosidase in a second infantile variant (217LAD; residual activity, 0.3%) was very similar, although the molecular defect was shown to be different. In this case, the 110-kD precursor was detectable via both immunoblotting (pattern as for 371 LAD, Fig. 3) and immunoprecipitation (14). However, the

1. *Abbreviation used in this paper:* RER, rough endoplasmic reticulum.

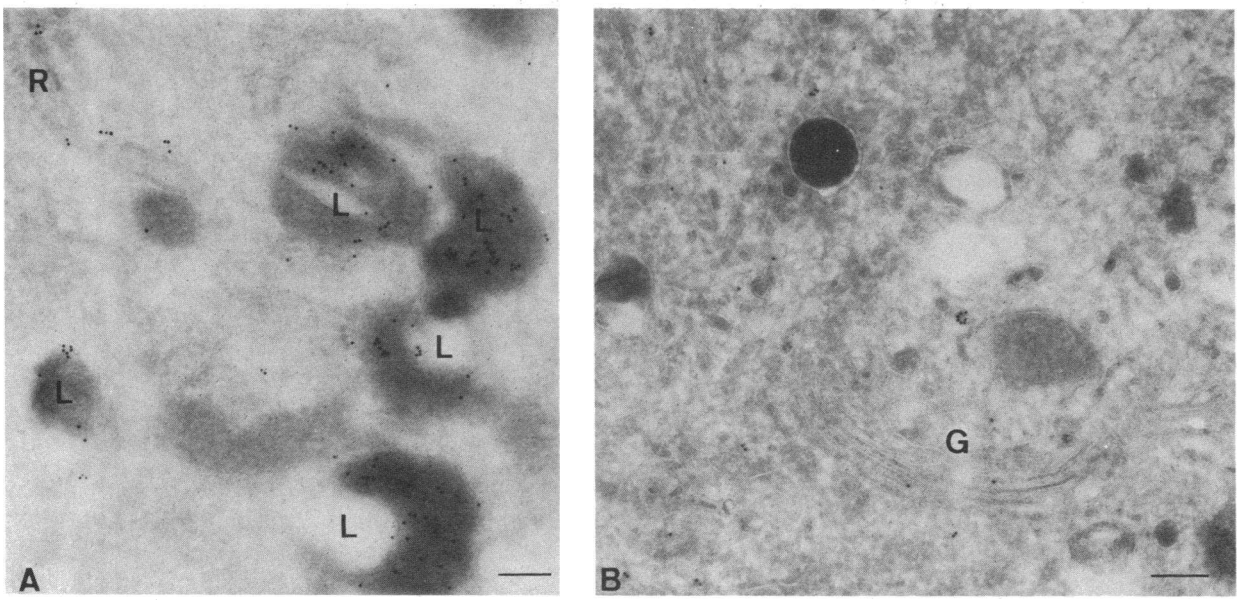

Figure 4. Subcellular localization of acid α-glucosidase in fibroblasts from healthy individuals. Ultrathin frozen sections of fibroblasts were incubated with antibodies against acid α-glucosidase, and subsequently with goat anti-rabbit IgG coupled to colloid gold particles of 10 nm. They were studied with a Philips 400 electron microscope at 80 kV. Bar, 0.1 μm. (*A*) Localization of acid α-glucosidase in rough endoplasmic reticulum (*R*) and lysosomes (*L*). (*B*) Localization of acid α-glucosidase in Golgi complex (*G*).

precursor of this mutant cell line has no phosphorylated mannose residues (14), whereas phosphorylation is considered to be essential for the transport of lysosomal enzymes to the lysosomes via the mannose 6-phosphate receptor (32, 34). Also labeled lysosomes were difficult to find in this mutant, but the RER was weakly labeled, as in control cells (Fig. 6 *C*). Lysosomes with significantly more gold particles were encountered in cell line 75RD100, which showed trace amounts of 95 and 76-kD enzyme protein on immunoblots (Fig. 6 *D*). The residual activity (0.5%) was, however, very low. Interestingly, most of the lysosomal label appeared to be membrane associated. Semiquantitative data on the lysosomal labeling are presented in Fig. 5.

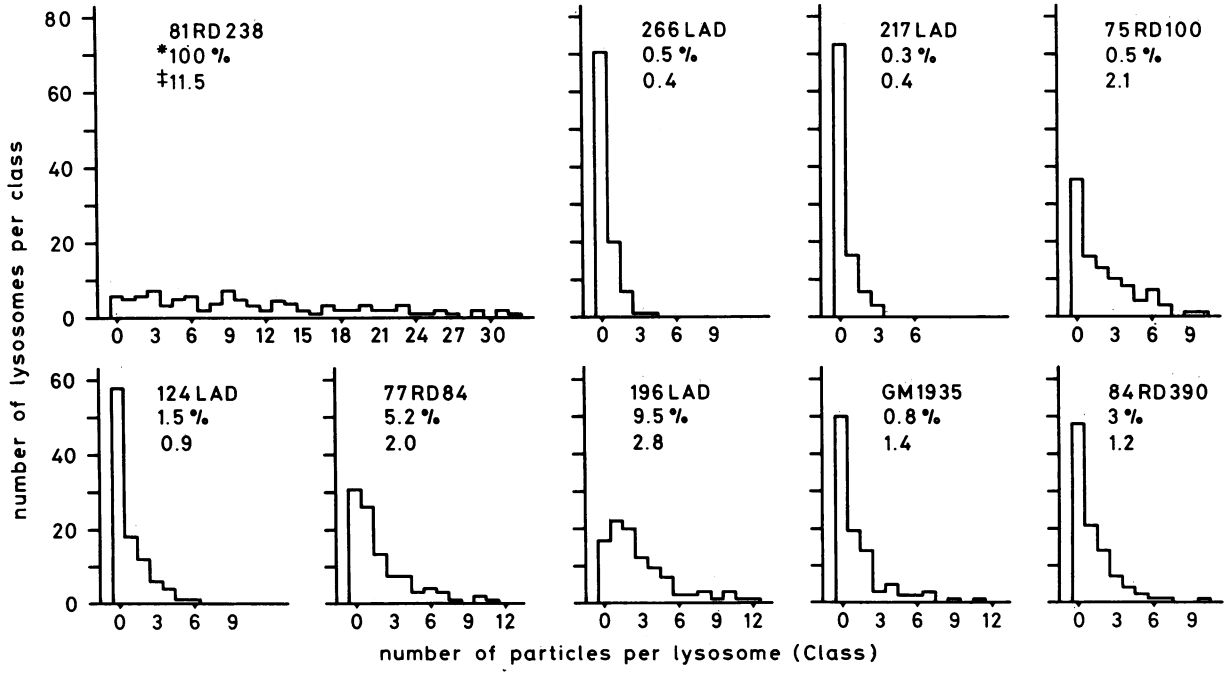

Figure 5. Semiquantitative data on the labeling of lysosomes in fibroblasts from patients with glycogenosis type II in comparison with normal fibroblasts. Acid α-glucosidase was immunocytochemically labeled as described in Fig. 4. For each cell line, 100 lysosomes were randomly chosen and the number of gold particles per single lysosome was counted. Lysosomes with the same number of particles were assembled in one class, and the number of lysosomes per class was determined. *Acid α-glucosidase activity of the cell lines is given as percentage of the activity of cell line 81RD238. ‡Indicates average number of gold particles per lysosome.

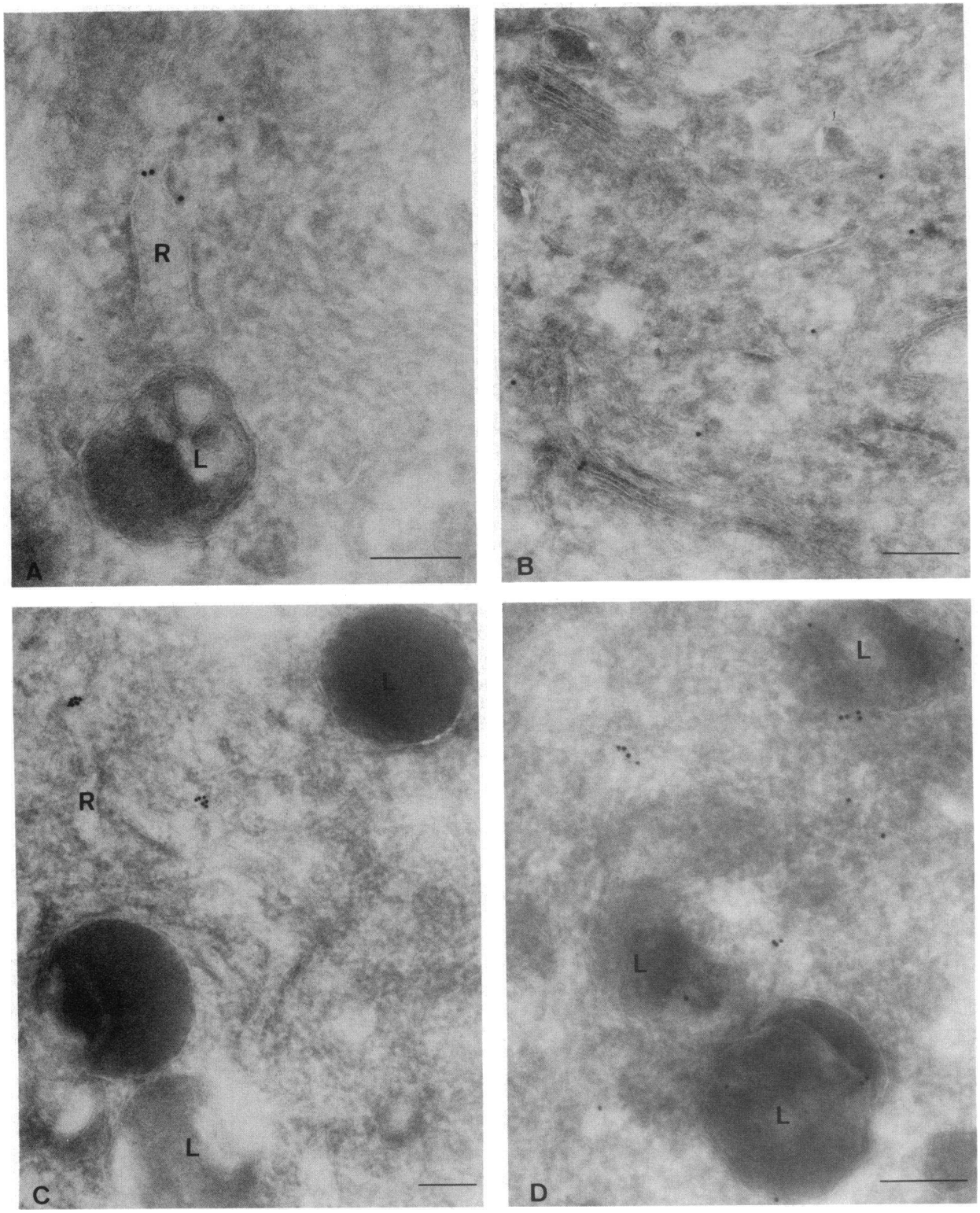

Figure 6. Subcellular localization of acid α-glucosidase in fibroblasts from patients with infantile glycogenosis type II. For methods, see legend to Fig. 4. Acid α-glucosidase is observed in (*A*) rough endoplasmic reticulum (*R*) and (*B*) Golgi complex, but not in (*A*) lysosomes of cell line 266LAD. (*C*) Lysosomes of cell line 217LAD are also devoid of label, but (*D*) lysosomes with membrane associated label are seen in cell line 75RD100. Bar, 0.1 μm.

Two of the patients who were designated as juvenile variants (124LAD and 77RD84) but differed in residual enzyme activity and molecular defect were studied. In cell line 124LAD (residual activity, 1.5%, Fig. 7 *A*) unlabeled lysosomes were more frequently found than in cell line 77RD84 (residual activity, 5.2%, Figs. 5 and 7 *B*). Labeling of the RER and the Golgi complex

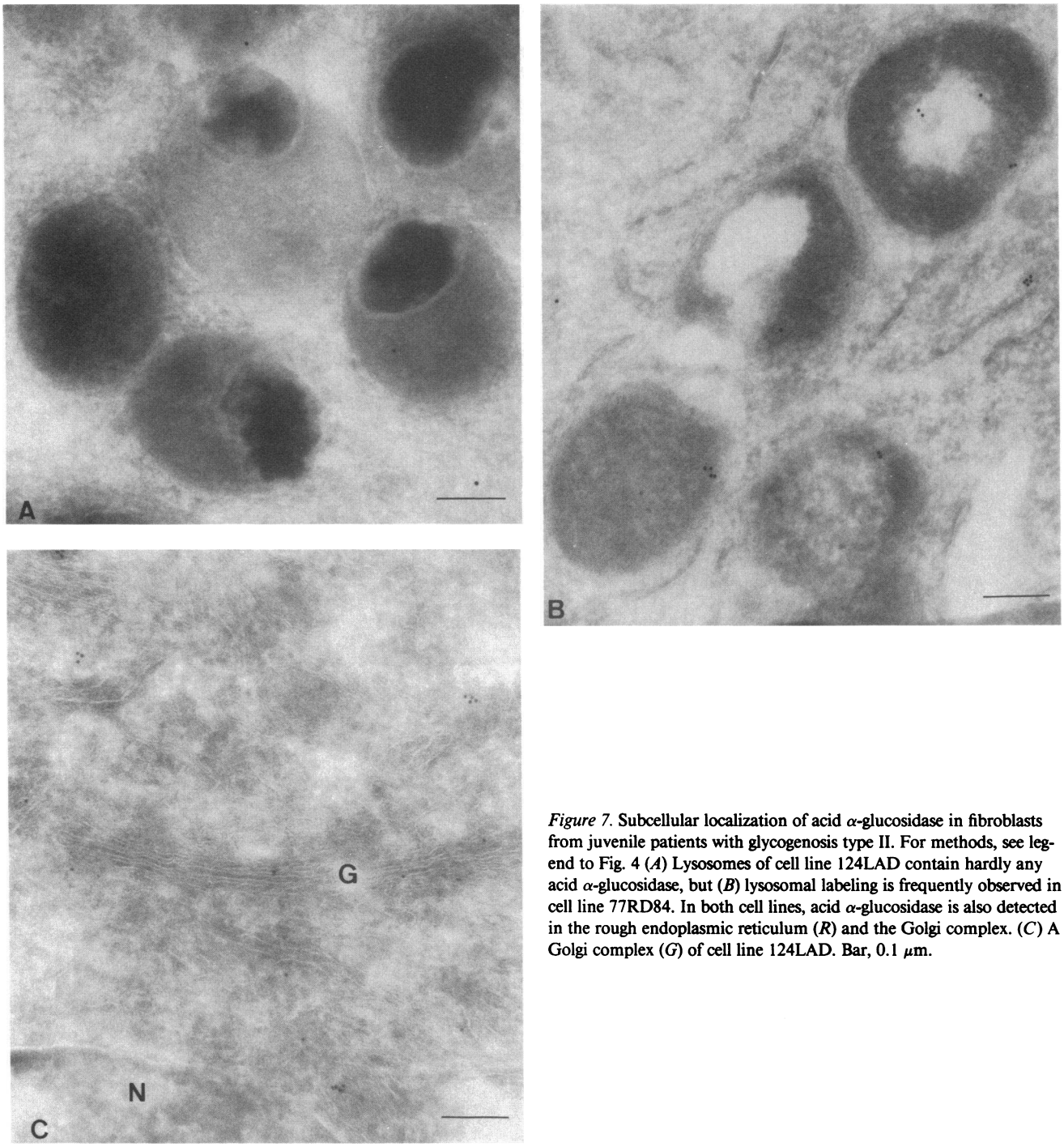

Figure 7. Subcellular localization of acid α-glucosidase in fibroblasts from juvenile patients with glycogenosis type II. For methods, see legend to Fig. 4 (*A*) Lysosomes of cell line 124LAD contain hardly any acid α-glucosidase, but (*B*) lysosomal labeling is frequently observed in cell line 77RD84. In both cell lines, acid α-glucosidase is also detected in the rough endoplasmic reticulum (*R*) and the Golgi complex. (*C*) A Golgi complex (*G*) of cell line 124LAD. Bar, 0.1 μm.

in both cell lines appeared normal compared with control (see Fig. 7 *C* for cell line 124LAD).

Fig. 8 is a composition of electron micrographs of fibroblasts from three adult patients. The nature of the mutation in each of these variants was shown to be different. Cell line 196LAD (immunoblotting pattern as for 79RD102, Fig. 3) had much higher residual activity (9.5%) than cell lines GM1935 (0.8%) and 84RD390 (3.0%), and this higher activity corresponded with the presence of mature forms of acid α-glucosidase. A phosphorylation defect was demonstrated in variant GM1935 (14), but phosphorylation was found to be normal in variant 84RD390 (A.J.J.R., unpublished results). Lysosomal labeling was frequently obtained in cell line 196LAD (Fig. 8 *A*) but much less often in GM1935 (Fig. 8 *B*) or in 84RD390 (Fig. 8 *C*). These observations are shown semiquantitatively in Fig. 5. Labeling of the RER of GM1935 appeared normal but the Golgi complex and its associated vesicular structures seemed to be labeled unusually strongly, when compared with several Golgi areas in normal and mutant cells (Fig. 8 *D*). Unfortunately, semiquantitative data on the Golgi labeling could not be obtained. The morphology of the Golgi compartment with its cisternae and many associated vesicular structures made this possible.

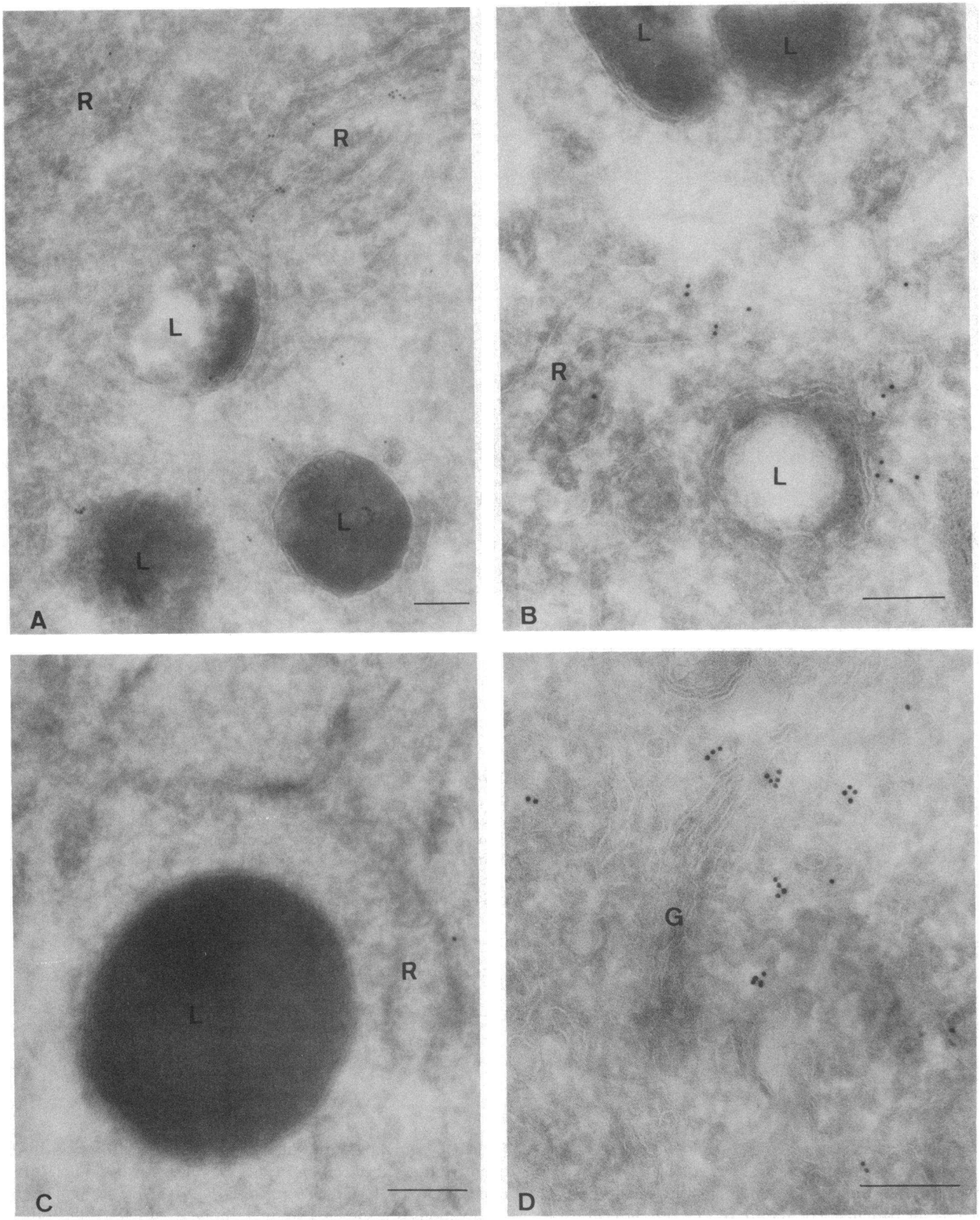

Figure 8. Subcellular localization of acid α-glucosidase in fibroblasts from adult patients. For methods, see legend to Fig. 4. (*A*) Lysosomal acid α-glucosidase is seen in cell line 196LAD, but rarely in GM1935 (*B*) or 84RD390 (*C*). Gold-labeled acid α-glucosidase is also detectable in the rough endoplasmic reticulum (*R*) (see [*A*] for cell line 196LAD) and in the Golgi complex (see [*D*] for cell line GM1935). Furthermore, concentrations of label are found in cytoplasmic areas of cell line GM1935 (*E*). Bar, 0.1 μm.

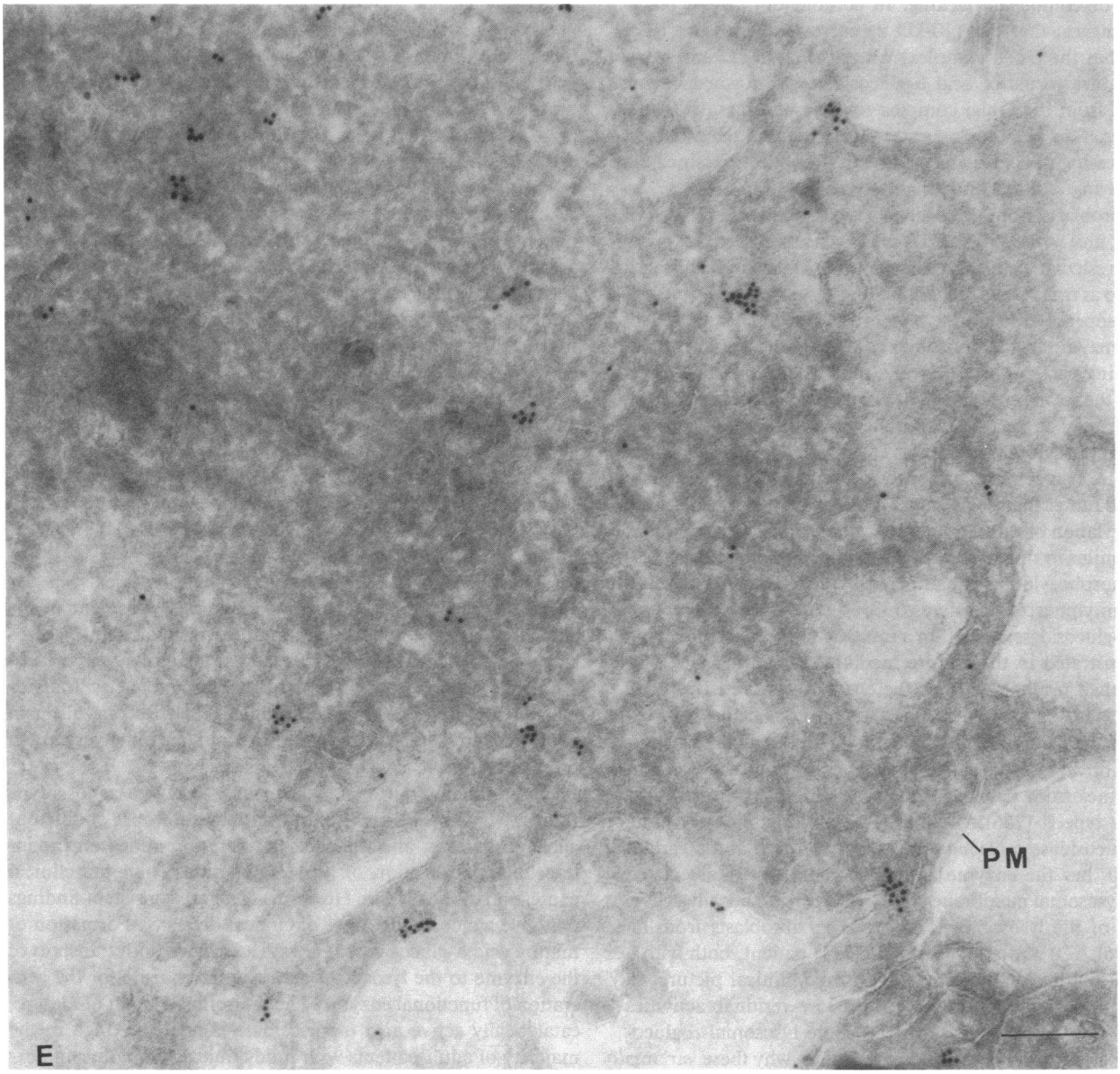

Figure 8 (*Continued*)

A characteristic feature of mutant GM1935 was labeling of the cytoplasm, often in the vicinity of the plasma membrane (Fig. 8 *E*). It was unclear with what type of cellular structure the label was associated.

Discussion

The cause of clinical diversity in glycogenosis type II was investigated by collecting detailed information on the biosynthesis, intracellular localization, and catalytic activity of mutant acid α-glucosidase in cultured fibroblasts from a large series of patients. The results obtained with the pulse-chase labeling or immunoblotting procedure turned out to be slightly different. The long-lived mature forms of acid α-glucosidase (70 and 76 kD) with an estimated half-life of 8–15 d (20) are quantitatively underestimated compared with precursor forms in relatively short pulse-chase periods. The immunoblotting procedure gives, in this respect, more accurate information on the actual amount of the various molecular species of acid α-glucosidase in the "steady state," and was chosen for comparing clinical variants.

A prominent finding is the reduced amount of mature enzyme protein in all 30 mutant cell lines. In view of the present results it is likely that the reduction of cross-reactive material, measured by Beratis et al. (11) in 10 of 11 cases of glycogenosis type II, is also due to deficiency of mature acid α-glucosidase. Variants with a normal production of enzyme may be more common in Japan (16). These findings indicate that mutations at the acid α-glucosidase locus easily disturb the biosynthesis and transport of this enzyme and perhaps reduce its stability in prelysosomal or lysosomal compartments.

Immuno–electron microscopy confirms this view and gives additional information on the fate of mutant enzyme. The RER and Golgi complex of all mutant cell lines are normally labeled, but the lysosomes are deficient in acid α-glucosidase. At the same time, the 110-kD precursor is the only molecular form of acid α-glucosidase that is detectable in all the various mutants

by immunoblotting, whereas the 95, 76, and 70-kD forms are partially or completely lacking. In combination, these observations suggest that the 110-kD precursor traverses the RER and reaches the Golgi complex, whereas the 95-kD and other more mature forms of acid α-glucosidase are formed during transport from the Golgi complex to the lysosomes, or in the lysosomes. This view corroborates recent data on the transport and processing of acid α-glucosidase in fibroblasts obtained via pulse-labeling and cell fractionation studies (33).

In fibroblasts from glycogenosis type II patients (217LAD, 124LAD, and 84RD390), acid α-glucosidase seems to disappear from the vesicular elements at the trans side of the Golgi complex, referred to as trans Golgi reticulum. Only a fraction of the mutant enzyme appears to reach the lysosomes. Increased turnover of mutant enzyme in the lysosomal compartment would explain this labeling pattern. Alternatively, mutant enzyme may be diverted to the wrong compartment because of a processing defect. The latter may be the case in variant GM1935 where abnormal labeling of the cytoplasm is observed, especially near the plasma membrane. It is tempting to speculate that acid α-glucosidase is secreted rather than sequestered in lysosomes, due to deficient phosphorylation of this mutant (14). Indeed, the labeling pattern is very similar to that of acid α-glucosidase in I-cell disease (29) where phosphorylation is hampered by deficiency of N-acetylglucosaminylphosphotransferase (32, 34). However, no secretion of acid α-glucosidase, at least in a catalytically active form, could be demonstrated in the culture medium of cell line GM1935 (unpublished results), whereas the enzyme is secreted in I-cell medium.

Comparing the amount of labeling in different clinical variants, it is obvious that the fewest enzyme molecules are present in lysosomes from patients with severe infantile forms of glycogenosis type II (266LAD and 217LAD). Significantly more acid α-glucosidase molecules are detectable in infantile variant 75RD100, but the enzyme appears predominantly associated with the lysosomal membrane and is hardly enzymatically active. Labeling of the lysosomes is strongest in fibroblasts from an adult (196LAD) and a juvenile (77RD84) patient, both with a relatively high residual activity and a mild clinical picture. In the remaining cell lines with rather low residual activities (84RD390, GM1935, and 124LAD) some lysosomal α-glucosidase is detectable. This may in part explain why these variants do not present with a severe infantile form of glycogenosis type II despite the fact that mature enzyme is hardly detectable, if at all, on immunoblots.

The molecular heterogeneity, even within clinical subtypes, is striking. At least four different mutant phenotypes can apparently lead to the infantile form of the disease. The first type of mutation, described by Beratis et al. (19) and Ninomiya et al. (16) but not encountered here, allows the formation of a normal quantity of enzyme protein but in a catalytically inactive form. The type of mutation present in cell line 372LAD and in three other infantile cases leads to a partial deficiency of mature enzyme with a concomitant profound deficiency of catalytic activity (Table I). The remaining cell lines from infantile patients are completely devoid of mature acid α-glucosidase, but they do produce the 110-kD precursor, as in 371LAD. This precursor is in most cases detectable via immunoprecipitation but in cell lines 371LAD, 79RD34, and 266LAD it has the peculiar characteristic that it is not detectable in this way. Because these three "patients" are sibs, it is almost certain that they carry the same mutant gene(s). A phosphorylation defect has been demonstrated in variant 217LAD (14), but the other infantile cases were not investigated in this way. Such an analysis might reveal further heterogeneity.

Two types of mutation are observed among juvenile variants. One gives rise to a low steady-state level of mature, catalytically active enzyme (77RD84), and the other interferes with the maturation of acid α-glucosidase from the 110-kD precursor on (124LAD and 82RD101). The residual activity in this clinical category varies from 2 to 5%.

Significantly higher residual activities, ranging from ~ 10 to 29%, are measured in fibroblasts from 13 out of 16 adult patients (Table I). Although still reduced, this higher level of activity is consistent with the expression of mature acid α-glucosidase. With the exception of mutant A.S., in which aberrant proteolytic processing is observed, the other 12 adult patients may have a similar type of mutation. The remaining three adult variants, GM1935, 84RD390, and E.M., are strikingly different because mature forms of acid α-glucosidase are virtually absent and the residual activities, measured with artificial substrate and glycogen, are even below those measured in juvenile forms of the disease. Here we are dealing with at least two types of mutation because the precursor of GM1935 is not phosphorylated (14), whereas there is no such defect in 84RD390 (A.J.J.Reuser, unpublished results).

Thus, at least 10 different mutant phenotypes exist in glycogenosis type II as counted by combining assessment of the clinical phenotype and the nature of the residual acid α-glucosidase polypeptides (Table II). Several of the individuals are undoubtedly heterozygous for two distinct mutant alleles. Individual 82RD167, for example, presumably carries one allele in common with 266LAD (his grandson) and one other very much more benign in its effect.

Summarizing our data, we conclude that glycogenosis type II is very heterogeneous with respect to the number of different allelic mutations, and the effect of these mutations on the intracellular localization of acid α-glucosidase and the clinical expression of the disease. However, there are consistent findings that give a clue to the cause of clinical diversity. Formation of mature acid α-glucosidase is always associated with transport of the enzyme to the lysosomes and is a prerequisite for the generation of functional enzyme. The lysosomal content of mature, catalytically active acid α-glucosidase is relatively high in the majority of adult patients with mild clinical symptoms and extremely low in severe infantile forms of the disease. This makes us believe that the clinical course of glycogenosis type II is pri-

Table II. Mutant Phenotypes in Glycogenosis Type II

Clinical subtype	Mature acid α-glucosidase	Precursor acid α-glucosidase	
		Immunoprecipitable	Phosphorylated
Infantile	Present, CRM reduced	yes	ND
	Undetectable	yes	no
		no	ND
Juvenile	Present, CRM reduced	yes	yes
	Undetectable	yes	yes
Adult	Present, CRM reduced	yes	yes
	Present, Abnormal M_r	yes	ND
	Undetectable	yes	yes, no

CRM, cross-reactive material; ND, not determined.

marily determined by the amount of functional acid α-glucosidase. However, the fact that some patients with a relatively mild phenotype have little enzyme in the lysosomes is puzzling. An explanation could be that the amount of functional enzyme is somewhat higher in the critical organs, heart, and skeletal muscle, due to subtle tissue differences in factors that affect the processing and intracellular transport of these particular mutant enzymes. Metabolic factors are likely to play a secondary role in the pathogenesis of the disease.

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