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DEFICIENT LYSOSOMAL CARBOXYPEPTIDASE ACTIVITY IN GALACTOSIALIDOSIS

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In the lysosome, the glycosidases neuraminidase (EC 3.2.1.18) and ß-galactosidase (EC 3.2.1.23) are associated to a 52 kDa "protective protein" to form a large multi-enzymatic complex. Deficient synthesis or inactivation of this protective protein causes galactosialidosis, a lysosomal storage disorder in man in which both neuraminidase and ß-galactosidase activities are deficient. Since the protective protein possesses extensive sequence homology with carboxypeptidase Y (carb Y) and the KEX 1 gene product from yeast, we have used the artificial substrate N-CBZ-Phe-Leu to detect and characterize the peptidase activity of the lysosomal carboxypeptidase (carb L). Using both a purified preparation of the lysosomal multi-enzymatic complex and cultured skin fibroblasts of patients affected with galactosialidosis, we demonstrate that the 52 kDa protective protein is responsible for carb L activity. The fibroblasts of three patients affected with late infantile and juvenile galactosialidosis were found to be deficient in carb L activity (1.4% of normal mean value).

The "protective protein" is part of a multi-enzymatic lysosomal complex (MEL-complex) with neuraminidase (acylneuraminyl hydrolase, EC 3.2.1.18) and ß-galactosidase (galactosyl hydrolase, EC 3.2.1.23) in which it is required for aggregation of the complex, stabilization of ß-galactosidase and expression of neuraminidase activity (1-3). Because of its role in the MEL-complex, defective or absent protective protein leads to secondary deficiency of both neuraminidase and ß-galactosidase activities in lysosomes (3,4). This causes galactosialidosis, a lysosomal storage disorder characterized by the accumulation of various oligosaccharides and gangliosides in tissues, progressive cerebelar ataxia, myoclonus, gait disturbance and the presence of macular cherry-red spot (4,5). The existence of different forms of galactosialidosis with variable levels of residual enzyme activities and different clinical manifestations (4,6) prompted us

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to devise a simple and direct activity test based on the primary defect: the deficiency of the protective protein (1,4).

The cDNA of the 52 kDa "protective protein" has been recently cloned and the deduced amino acid sequence (7) was found to be homologous to those of carboxypeptidase Y (carb Y) (8) and KEX 1 gene product from yeast (9). The protein is a heterodimer made of equimolar quantities of 32 and 20 kDa protomers linked together by a disulfide bridge (3,7). The serine 150 residue is in the active site of carb Y (10) and likely has the same function in the lysosomal carboxypeptidase/protective protein (carb L). In addition, it was previously suggested that a histidine residue in carb Y may participate in a charge-relay mechanism of peptide or ester hydrolysis (11). We exploited this information to demonstrate that the 52 kDa heterodimer is responsible for the carb L activity and devised a simple assay system to determine its activity. We report that carb L activity is deficient in cultured skin fibroblasts of patients affected with late infantile and juvenile galactosialidosis.

MATERIAL AND METHODS

Purification of the multi-enzymatic lysosomal complex. The MEL-complex was purified from human placenta obtained at caesarean section by a modification of the method of Verheijen et al. (2) as described by Potier et al. (12). The specific activities of neuraminidase and ß-galactosidase in the purified preparation were 160 and 10,550 nmoles/min/mg of protein, respectively, as assayed with the corresponding 4-methylumbelliferyl-glycoside derivatives as substrates (13,14). Proteins were assayed according to Bradford (15) or Lowry et al. (16) using bovine serum albumin as standard.

Carboxypeptidase assay. The carboxypeptidase activity of the purified MEL-complex, cultured skin fibroblasts and a commercial carb Y preparation (Sigma) was assayed according to a modification of the method of Stevens et al. (17). The enzyme preparation was incubated at 25°C in 50 mM acetate buffer at either pH 5.5 or 5.75 containing 0.75 mM N-CBZ-L-phenylalanine-L-leucine (N-CBZ-Phe-Leu) as substrate in a total volume of 100 µl. After 30 min, 100 µl H_2O was added and the reaction was stopped by boiling for 3 min. The liberated L-leucine was determined colorimetrically with an L-amino acid oxydase-peroxydase-o-dianisidine reagent (17).

Active site reagents. The active site serine reagent phenylmethylsulfonyl fluoride (PMSF) was preincubated for 60 min at 25°C with 0.02 µg of the purified MEL-complex or 0.01 µg of carb Y in presence of 20 mM sodium acetate buffer (pH 5.75), NaCl 100 mM in a total volume of 50 µl. The histidine reagents chloromethylketone derivatives of N-CBZ-L-phenylalanine and N-α-tosyl-L-lysine, the thiol reagent iodoacetamide and the chelating agent EDTA were used under the same experimental conditions as above except that with

iodoacetamide the incubation time was 120 min and the amounts of MEL-complex or carb Y were 0.2 or 0.1 μg, respectively. At the end of preincubation with the inhibitors, 50 μl of 1.5 mM N-CBZ-Phe-Leu substrate was added to the incubation medium to determine the enzymatic activity as described above. Appropriate controls were run to ensure that the reagents and inhibitors did not interfere with the enzymatic determination of the liberated L-leucine (17).

High performance liquid chromatography. High performance liquid chromatography of standard proteins and purified MEL-complex was performed on a Waters Associates instrument model 6000 A according to le Maire et al. (18) using a 20 mM sodium acetate buffer (pH 5.2), 100mM NaCl with and without 0.5 M guanidinium hydrochloride as a dissociating agent. The chromatographic column was a Waters Associates SW 300 gel-filtration type eluted at a flow rate of 0.8 ml/min. The following standard proteins with known Stokes' radii (R_s) were used to calibrate the column: thyroglobulin (R_s, 8.6 nm), ferritin (6.3 nm), aldolase (4.6 nm), albumin (3.5 nm), ovalbumin (2.8 nm), trypsin inhibitor (2.2 nm) and ribonuclease A (1.75 nm). Usually, 120 μg of MEL-complex were injected into the column and 200 μl fractions were collected for carb L assay. Analysis of the protein peaks separated on the HPLC column was done by SDS-polyacrylamide gel electrophoresis under reducing conditions according to Laemmli (19). For HPLC under dissociating conditions, the MEL-complex at 0.9 mg/ml was denatured for 4 h at 4°C in the acetate buffer mentionned above containing 0.5 M guanidinium-HCl (GuHCl).

Cultured skin fibroblasts. Skin fibroblasts of three patients affected with the late infantile and juvenile forms of galactosialidosis, and controls were cultured in minimum essential medium supplemented with 10% fetal calf serum in absence of antibiotics. One patient cell line came from our own cell bank and two lines were obtained from Dr. John Callahan (University of Toronto). The three fibroblast lines used contained less than 13% normal neuraminidase and ß-galactosidase activities but normal N-acetyl-hexosaminidase activity. At confluence, the cells were scrapped, washed and homogeneized in water with a 15 sec burst from a sonicator. Carb L activity was determined in the fibroblast homogenate as described above.

RESULTS AND DISCUSSION

With N-CBZ-Phe-Leu as substrate, the carb L associated with the purified MEL-complex showed maximum activity at pH 5.5 in sodium acetate buffer (data not shown). The more acidic pH optimum of carb L as compared to carb Y (pH optimum, 6.0-6.5) (10,20) was expected if carb L exerts its catalytic function in the acidic environment of the lysosome. The K_m value was 0.42 mM and the V_{max} was 1160 nmole/min/mg of purified MEL-complex proteins. At concentrations higher than 0.75 mM, we observed a substrate inhibition effect similar to that reported for carb Y (20).

Table 1. Effect of active site reagents and protease inhibitors on carboxypeptidase L and Y activities

Reagent	Reactive residue	Reagent concentration (mM)	Enzyme activity (% of control)	
			Carb L	Carb Y
PMSF	Ser	0.5	52	2
		1.0	35	0
ZPCK	His	0.1	62	65
		0.5	36	35
TLCK	His	0.1	84	73
		0.5	76	58
Iodo-acetamide	Cys, His Lys	20	16	85
EDTA	-	1.0	105	89
		10	105	91
leupeptin	-	0.5	92	81
		2.0	84	61

PMSF: Phenylmethylsulfonylfluoride, ZPCK: N-CBZ-L-phenylalanine chloromethylketone, TLCK: N-α-tosyl-L-lysine chloromethylketone, EDTA: ethylenediaminetetraacetate.

The cell and particularly the lysosome contains a variety of proteases which may interfere with carb L assay. To rule out this possibility, we compared the effects of various protease inhibitors on carb Y and carb L activities (Table 1). Carb Y is a serine protease with a histidine residue in the active site (11). As can be expected from its homology with carb Y, carb L reacts with PMSF but its serine residue was less reactive than that of carb Y. The histidine reagents ZPCK and TLCK both affected the activities of carb L and Y to about the same extent. Iodoacetamide caused greater inhibition of carb L than carb Y. A reactive Cys residue probably near the active site of carb Y has been invoked to explain the inhibitory effect of iodoacetamide (21). Perhaps a similar phenomenon also takes place with carb L. Finally, leupeptin and EDTA were found to have no effect on carb L activity ruling out the possibility that carb L is a thiol-or a metalo-protease (22).

HPLC gel-filtration analysis of the purified MEL-complex revealed the presence of three protein peaks (Fig. 1A) corresponding to different constituents of the MEL-complex in a dynamic

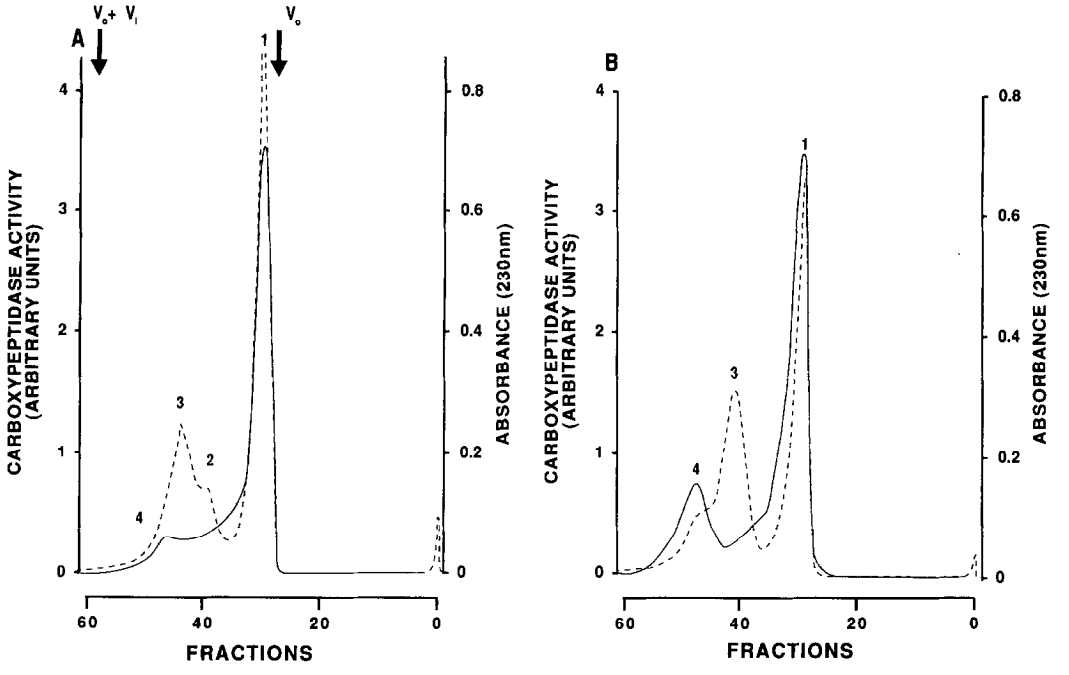

Fig. 1. HPLC gel-filtration analysis of the purified MEL-complex on a SW 300 column in absence (A) and in presence (B) of 0.5 M guanidinium hydrochloride. V_o = void volume, V_o + V_i = void volume plus volume inside the gel beads. Dashed line, absorbance at 230 nm; solid line, carboxypeptidase L activity.

equilibrium (12). The peak 1 is the largest molecular aggregate with a M_r of 679 kDa whereas peaks 2 and 3 represent tetrameric and dimeric forms of ß-galactosidase, respectively (12). Although all three peaks contain ß-galactosidase activity, both the neuraminidase and carb L activities were concentrated mainly in peak 1 (only carb L activity is shown in Fig. 1A). SDS-polyacrylamide gel electrophoresis of each peak separated by HPLC (Fig. 2A) revealed that the 32 and 20 kDa protomers (the two protomeric components of the 52 kDa heterodimer) are found mainly in peak 1 whereas peaks 2 and 3 contained relatively small amounts of these protomers (12). Therefore, the distribution of 32 and 20 kDa protomers in the peaks separated by HPLC fits well with the distribution of carb L activity (Fig. 1A). Other major protomeric components of the MEL-complex (Fig. 2) have also been previously described: the 76 kDa band is an IgM heavy chain which copurifies with the MEL-complex (3), the 66-63 kDa diffuse band is a mixture of neuraminidase and ß-galactosidase protomers (3,12), and the 54-52 kDa band is the unprocessed precursor of the heterodimer (1,7).

Treatment of the MEL-complex with 0.5 M GuHCl followed by HPLC analysis showed a displacement of the equilibrium between

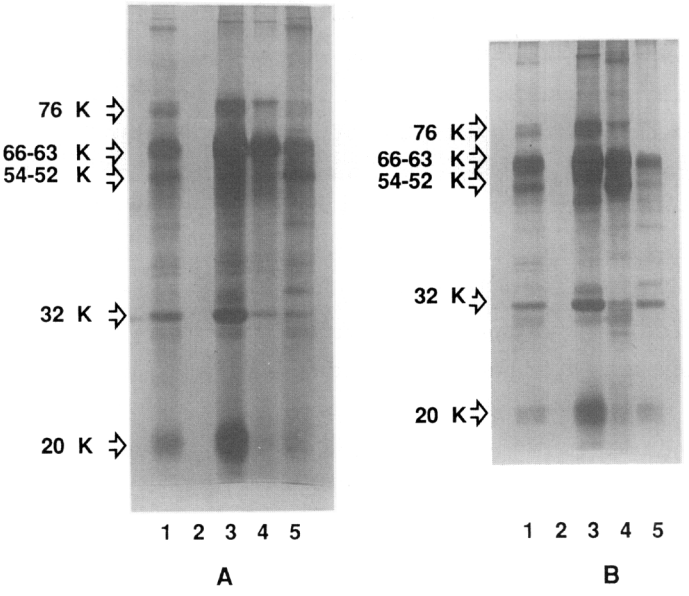

Fig. 2. SDS-polyacrylamide gel electrophoresis of the peaks separated on the HPLC-column. The gel concentration was 11 % (W/V) and the protein bands were revealed by a silver stain (23). The numbering of peaks corresponds to that in Fig. 1. (A) The MEL-complex under non-denaturing conditions: lane 1, 15 μg of MEL-complex protein; lane 2, buffer control; lane 3, peak 1 (37 μg); lane 4, peak 2 (10 μg) and lane 5, peak 3 (19 μg). (B) The MEL-complex in presence of 0.5 M GuHCl: lane 1, 15 μg of MEL-complex protein; lane 2, buffer control; lane 3, peak 1 (45 μg); lane 4, peak 3 (35 μg); and lane 5, peak 4 (10 μg). Dashed line, absorbance at 230 nm; solid line, carboxypeptidase L activity.

MEL-complex species towards lower molecular mass forms. Peak 1 decreased, peak 2 disappeared, peak 3 increased and a new species, peak 4 (shoulder on peak 3, Fig. 1B), was formed (Fig. 1A). The new species had a R_s of 3.5 nm, identical to that of bovine serum albumin and the carb L activity determined in the fractions eluted from the HPLC column seems associated to it (Fig. 1B). Analysis by SDS-polyacrylamide gel electrophoresis of peak 4 showed a higher content of the 32 and 20 kDa protomers (Fig. 2B). This finding supports the idea that the 52 kDa heterodimer is responsible for the carb L activity in the MEL-complex. The low GuHCl concentration used ensured a 53% recovery of carb L activity on the HPLC column. By comparison, 79 % of the carb L activity applied to the column was recovered in the absence of GuHCl. Higher GuHCl concentrations were more effective in dissociating the MEL-complex but caused heavy loss of carb L activity.

Final demonstration that the 52 kDa heterodimer is the protein responsible for carb L activity was obtained by assaying

carb L activity in cultured skin fibroblasts of galactosialidosis patients who are deficient in the 52 kDa heterodimer (1,4). The carb L activity in cultured skin fibroblasts of control subjects was 53.6 +/- 21.5 nmoles/min/mg of protein (mean +/- SD, n = 6) whereas it was only 0.7 +/- 1.2 nmoles/min/mg of protein in the cells of three patients affected with late infantile form of galactosialidosis. These results allow us to conclude that our assay in the fibroblasts is specific for lysosomal carb L activity since as indicated by the low residual activity in patient cells (1.4 % of controls), other proteases did not interfere with the assay.

Taken altogether, the evidence indicates that (a) the carb L activity is associated to the 52 kDa protective protein in the MEL-complex and has similar properties to homologous yeast carb Y; (b) the method we developed for carb L assay is highly specific to carb L; and (c) may be useful to detect primary deficiency of carb L activity in the cells of patients affected with late infantile and juvenile forms of galactosialidosis. It remains to determine how carb L plays its role in the maintenance of the MEL-complex and in the activation of neuraminidase.

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