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PLASMA MEMBRANE-BOUND AND LYSOSOMAL ISOZYMES OF AMINO ACID NAPHTHYLAMIDASE

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SUMMARY

An isozyme of amino acid naphthylamidase was found in the plasma membrane fraction of cultured HeLa cells. It was very similar to lysosomal isozymes with respect to some kinetic properties but had different electrophoretic mobility. After treatment with proteolytic enzymes the plasma membrane-bound isozyme seemed to be converted to the isozymes residing in the lysosomes. The possible precursorship of the plasma membrane-bound isozyme for the lysosomal isozyme is discussed.

INTRODUCTION

Several molecular forms, i.e. isozymes, of amino acid naphthylamidase have been described in cultured human cells [1, 2]. The isozyme differentiation has been suggested to be due to the association of amino acid naphthylamidase activity to lipid membranes of different subcellular organelles [3]. One isozyme was a soluble, cytoplasmic enzyme, which differed from microsomal and lysosomal isozymes with respect to some inhibitors and activators, molecular weight relationship etc. [2]. The microsomal and lysosomal isozymes were very similar except for solubility properties and electrophoretic mobility. Therefore it was proposed that they were the product of the same structural gene. This was supported by the fact that in some cultured glia and glioma cell lines both isozymes either were present or absent [4].

Beckman and Lundgren [5] showed that amino acid naphthylamidase activity was inducible by glucocorticoids. The kinetics of induction made it probable that the microsomal isozyme was a precursor of the lysosomal isozymes [6]. It is well known that lysosomal enzymes originate in the microsomal fraction, either from the endoplasmic reticulum or the Golgi apparatus [7]. The glucocorticoid induction of amino acid naphthylamidase isozymes might be an example of this. Therefore it was decided to further study the localization of the microsomal isozyme and characterize its transformation to the lysosomal form.

MATERIALS AND METHODS

HeLa cells were cultivated and harvested as described in a previous publication [2]. Homogenization was performed in 0.25 M sucrose, pH 7.0, at 4 °C with a tight

fitting Dounce homogenizer until nearly complete homogenization was achieved as judged by phase contrast microscopy. Homogenization was stopped when 5–10% of the cells still were intact as further homogenization meant irreproducible results.

Differential centrifugation was performed as described previously [3]. The cell debris and nuclear fraction obtained after centrifugation at 800 × *g* for 10 min was discarded. A combined mitochondrial and lysosomal fraction (M+L fraction) was obtained after centrifugation at 26 000 × *g* for 10 min. A microsomal fraction (the P fraction) was obtained after removal of the M+L fraction by centrifugation at 102 000 × *g* for 60 min. The resulting final supernatant (S fraction) and the M+L fraction were stored at −20 °C until enzyme analyses were performed. The microsomal P fraction was further subfractioned by making it 45% with respect to sucrose and incorporating it in a discontinuous sucrose gradient according to Fig. 1 in a 4-ml polyallomer tube. The gradient centrifugation was performed in the SW 56 rotor of a Beckman L2 65 B centrifuge at 70 000 × *g* for 16 h. After centrifugation 15 subfractions were removed by aspiration from above. All fractions and subfractions were stored at −20 °C until enzyme analyses were performed. Enzyme solubilization was achieved by 10 cycles of freezing and thawing or treatment with Triton X-100 before electrophoresis, according to a previous description [2]. In order to separate the lysosomal microsomal isozymes [2], a M+L or a P fraction was frozen and thawn 10 times and then centrifuged at 102 000 × *g* for 60 min. The 102 000 × *g* supernatant of the M+L fraction was considered to represent the lysosomal, easily releasable isozymes, while the 102 000 × *g* pellet of the P fraction represented the microsomal membrane bound isozyme.

For morphological examination, Subfractions 6–9 and 13–15 respectively were pooled, diluted with 0.25 M sucrose and centrifuged at 50 000 × *g* for 30 min. The pellets were fixed and embedded according to Nachman et al. [8]. Sections were done from different parts of the pellets, as in some cases there were differences between separate levels. Staining was performed with uranyl acetate and lead citrate and the sections were examined in a Zeiss EM9 electron microscope.

Polyacrylamide gel electrophoresis was performed according to Williams and Reisfeld [9] with the modification described earlier [2]. No difference was found with respect to resolution between samples of pH 5.5 or 7.0. Molecular weight relationships were estimated by Ferguson plots with some modifications [10, 11, 2].

For treatment of the P fraction with enzymes, buffered enzyme solutions were made in the following way: 100 μg enzyme was solubilized in 1 ml 0.05 M Tris–phosphoric acid buffer, pH was adjusted to 5.5 when neuraminidase and papain was used and to 7.0 when trypsin and phospholipase C was used. Papain was activated by 5 mM cystein. Equal proportions of buffered enzyme solution and the 102 000 × *g* pellet at the P fraction were mixed and incubated at 37 °C for 1 h, when not otherwise indicated. Before electrophoresis pH was adjusted to 5.5. Trypsin digestion was stopped by addition of soybean trypsin inhibitor in a final concentration of 50 μg/ml. Neither trypsin digestion for 6 h, nor the addition of the trypsin inhibitor affected the total activity of amino acid naphthylamidase.

Amino acid naphthylamidase activity was assayed according to Lundgren [3]. In order to estimate substrate specificity the rate of hydrolysis of eight different amino acid β-naphthylamides in 0.68 mM concentration was assayed in 0.05 M phosphate buffer, pH 7.0. The K_m value was determined with L-leucyl-β-naphthylamide as sub-

strate in the concentration range 0.5–0.01 mM at pH 7.0. The following enzymes were assayed according to the references given: β-glucuronidase [12], glucose 6-phosphatase [3], alkaline phosphatase [13], 5-nucleotidase [14], acid phosphatase [3]. Thiamine pyrophosphatase and uridine diphosphatase were assayed in 0.05 M Tris-HCl buffer, pH 7.5, containing 5 mM enzyme substrate and 5 mM $MgCl_2$. In the assays of phosphatases reaction was stopped and inorganic phosphate released was measured according to Itaya and Ui [15].

RESULTS

Gradient centrifugation

Two broad peaks of enzyme activity were easily recognizable in the sucrose gradient after centrifugation (Fig. 1). Glucose 6-phosphatase, the marker enzyme for endoplasmic reticulum was concentrated in Subfractions 13–15, which was also enriched in β-glucuronidase, an enzyme with a dual localization both in the endoplasmic reticulum and in the lysosomes [16]. The morphological structures in this fraction had the appearance of rough and smooth membranes (Fig. 2a). Some occasional mitochondria and lysosomes were also found (Fig. 2b), but since acid phosphatase was not enriched entirely in these subfractions (Fig. 1e) but was evenly distributed in both subfractions, the lysosomal contamination seemed to be without importance for interpretation of data concerning enzyme localization in the microsomal fraction.

In Subfractions 6–9, peaks for alkaline phosphatase and 5-nucleotidase were found, marker enzymes for the plasma membranes of cultured HeLa cells [17]. However, the peak of alkaline phosphatase was broader than the peak of 5-nucleotidase. The reason for this is unclear. Electron microscopy revealed smooth vesicular membranes of different sizes in the subfractions corresponding to these enzymes (Fig. 2c). The activity of amino acid naphthylamidase was also concentrated in these subfractions, the broad peak corresponded most closely to that of alkaline phosphatase.

There are no accepted markers for the Golgi apparatus in cultured human cells although uridine diphosphatase has been used in HeLa cell homogenates [17]. This enzyme and thiamine pyrophosphatase, another Golgi-associated enzyme [18, 19], were evenly distributed in both enzyme peaks. No membrane fragments in the electron micrographs could unequivocally be claimed to be made up of Golgi vesicles or membranes.

These results indicate that the microsomal amino acid naphthylamidase activity resides in the plasma membrane and not in the endoplasmic reticulum and probably also not in the Golgi apparatus.

Electrophoresis

In agreement with earlier results [2, 3], three main isozymes of amino acid naphthylamidase were found in the three fractions obtained after differential centrifugation (Fig. 3). In the M+L fraction the Isozymes B1–B3 were released by repeated freezing and thawing. Isozyme B1 was very weak compared to Isozymes B2 and B3, and therefore Isozyme B1 was not further considered. Isozyme A was found in fraction S. In the P fraction Isozyme C was predominating, although traces of Isozymes B2 and B3 could be seen. Isozyme C was the only component in Subfractions 6–9 in

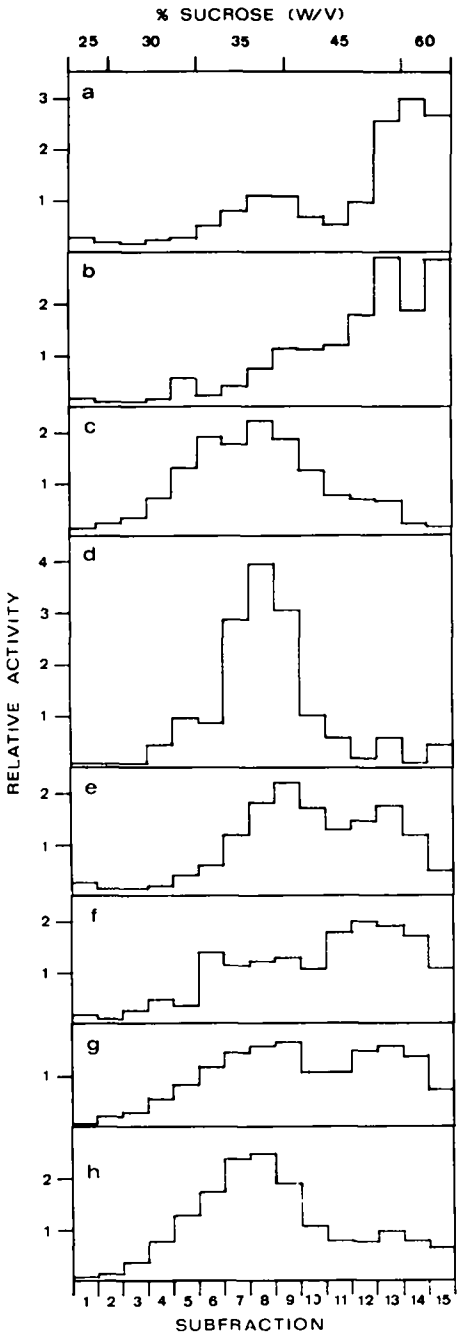

Fig. 1. Discontinuous sucrose gradient subfractionation of the P cell fraction a, glucose 6-phosphatase; b, β-glucuronidase; c, alkaline phosphatase; d, 5-nucleotidase; e, acid phosphatase; f, thiamine pyrophosphatase; g, uridine diphosphatase; h, amino acid naphthylamidase.

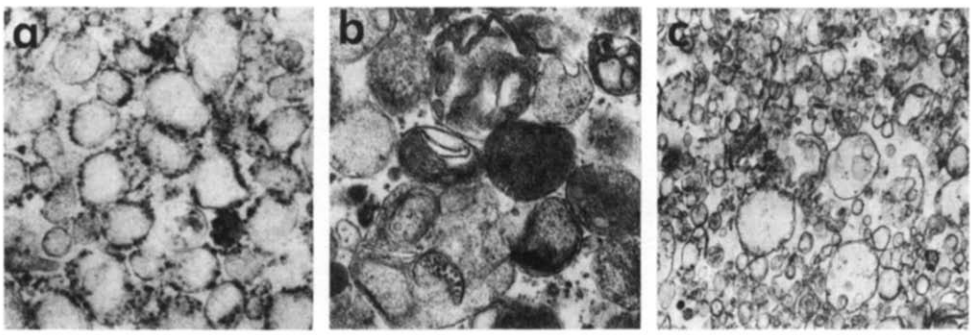

Fig. 2. (a) and (b) Ultrastructure of the pooled Subfractions 13–15 (× 46 000). (c) The pooled subfractions 6–9 (× 15 400). After pooling the subfractions were diluted and centrifuged. (a) and (b) represents different levels of the remaining pellets.

the gradient, while the lysosomal Isozymes B2 and B3 were found in Subfractions 13–15. Isozyme C penetrated into the gels only when the P fraction was treated with Triton X-100, otherwise it remained at the start.

Phospholipase C and trypsin treatment

Isozyme C from the P fraction was very sharp on the polyacrylamide gels, which may be due to membrane-derived hydrophobic residues, i.e. lipids, on the enzyme

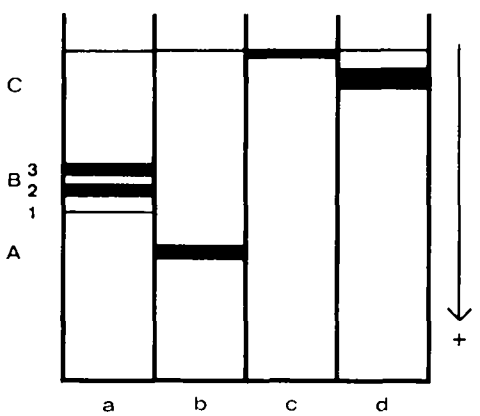

Fig. 3. Polyacrylamide gel electrophoresis of cell fractions. (a), M + L fraction; (b), S fraction; (c) P fraction; and (d), P fraction made 1% with respect to Triton X-100.

molecule. It was noted that the staining intensity of the traces of Isozymes B1 and B2 of the P fraction increased when the sample was subjected to temperatures above 37 °C (Fig. 4). Therefore the 102 000 × *g* pellet from a P fraction was incubated for 0–24 h at 37 °C and it is obvious from Fig. 4 that enzyme components appears similar to Isozymes B2 and B3. Therefore the possibility of removing membrane fragments from Isozyme C by enzyme treatment was tried. Phospholipase C did not affect the solubility or electrophoretic mobility of Isozyme C to any detectable degree. All the activity remained at the start, and after treatment with Triton X-100 an ordinary

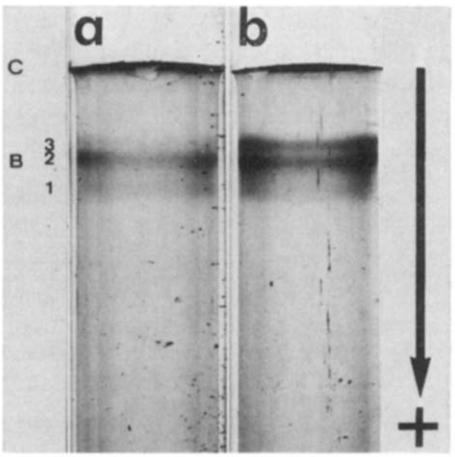

Fig. 4. The effects of heating on the 102 000 × *g* pellet of the P fraction. a, P fraction stored at 0 °C for 24 h; b, P fraction stored at 37 °C for 24 h. Triton X-100 was not added, therefore Isozyme C remains at the start.

Isozyme C appeared. However, the B isozymes which appeared during the incubation at 37 °C were slightly retarded compared to the controls.

Papain and trypsin digestion markedly reduced the staining intensity of the C isozyme while two enzyme components appeared with the same electrophoretic mobility as Isozymes B2 and B3 (Fig. 5). The papain treatment was not further studied as the enzyme components obtained were rather blurred, although the electrophoretic mobility was the same.

Thus there seemed to be a conversion of Isozyme C to Isozymes B2 and B3. This possibility was elucidated by the following experiments.

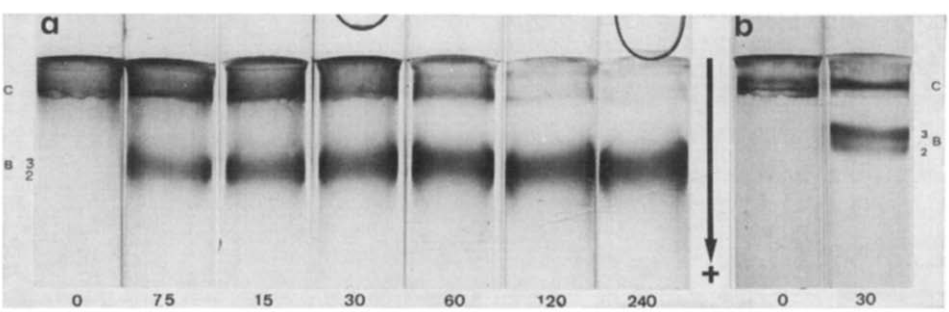

Fig. 5. The effect of trypsin on isozyme C in the 102 000 × *g* supernatant of the P fraction. The gels were made 1 % with respect to Triton X-100 when enzyme treatment was completed. a, trypsin treatment for 0–240 min; b, trypsin treatment for 30 min.

Neuraminidase treatment

After treatment of the 102 000 × *g* supernatant of the M+L fraction with neuraminidase the B components were slightly retarded (Fig. 6) on the gels, indicating a loss of sialic acid. There was no change of staining intensity of Isozymes B2 and B3, nor was there any increase of Isozyme C as judged by visual inspection. Isozyme A

in the S fraction was not affected in agreement with earlier results [20]. The C isozymes near the start were not possible to estimate, because of the very short migrating distance. But the two Isozyme B-like components derived from trypsin treatment of Isozyme C were also retarded to the same degree as Isozymes B2 and B3. Neuraminidase treatment was also conducted on the P fraction before treatment with trypsin,

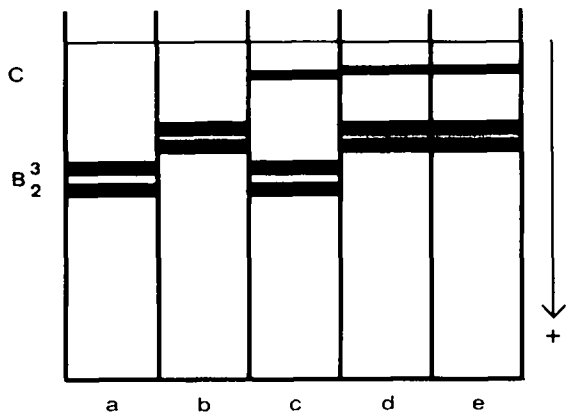

Fig. 6. The effect of neuraminidase treatment on Isozymes B2 and B3 in the M + L fraction and Isozyme C of the P fraction, prepared as described in Materials and Methods. All fractions were made 1 % with respect to Triton X-100 prior to electrophoresis. a, M + L fraction before and, b, after treatment with neuraminidase; c, trypsin-digested P fraction before and, d, after neuraminidase treatment; e, the same as d except that neuraminidase treatment was done before trypsin treatment.

however, the same degree of retardation was still seen (Fig. 6e). Therefore it seems most likely that sialic acid also is attached on Isozyme C. It was noted that the degree of retardation of Isozymes B2 and B3 and the Isozyme B-like components was very like the retardation of the Isozyme B-like components achieved by treatment with phospholipase C. It has been shown by Emmelot and Bos [21] that phospholipase C preparations may be contaminated by neuraminidase.

Kinetic studies

Lineweaver–Burk plots were made and an apparent K_m value of $6 \cdot 10^{-5}$ M was calculated for Isozymes B2, B3 and C (Fig. 7). The same value was estimated for Isozyme C after trypsinization. In contrast Isozyme A had an apparent K_m value of about $2 \cdot 10^{-5}$ M.

Isozymes B2 and B3 and Isozyme C also had a similar substrate specificity which was not significantly different from that of Isozyme C after trypsin treatment (Table I). All isozymes utilized the L-alanyl derivative most efficiently, followed by the L-leucine derivative. In contrast to Isozyme A the β-naphthylamides of the basic amino acids arginine and lysine were hydrolysed at a low rate. There was also a marked difference in the utilization of the prolyl-β-naphthylamide.

Molecular weight relationships

Ferguson plots were made with Isozymes B and C and Isozyme C after treatment with trypsin. The plots in Fig. 8 show that the B and C isozymes have a similar

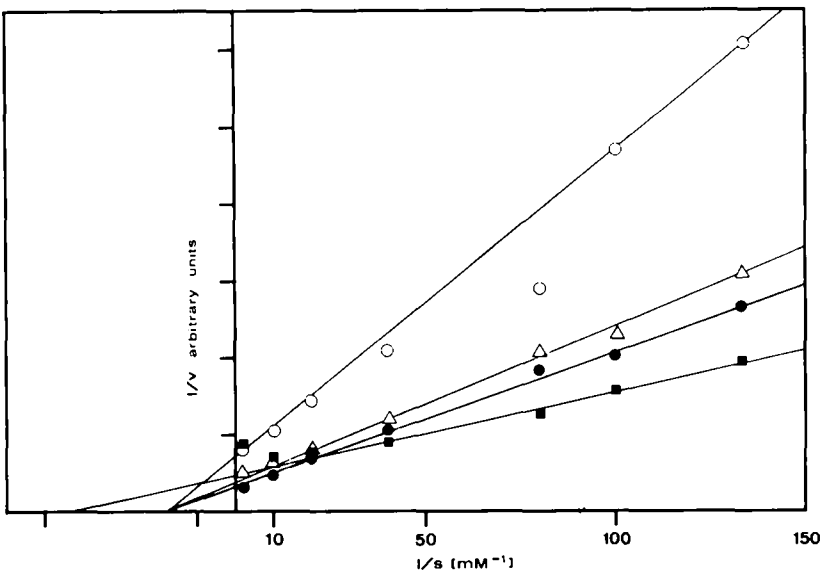

Fig. 7. Lineweaver–Burk plot of the isozymes of amino acid phosphatase. ●—●, isozymes B2 and B3 of the M + L fraction cell; △—△, Isozyme C of the P cell fraction; ○—○, Isozyme C of the P cell fraction, after treatment with trypsin; ■—■, Isozyme A of the S cell fraction.

slope indicating similarities in molecular weights but differences in charge. After treatment with trypsin, Isozyme C was much weaker, but had the same mobility as before. The new components appearing after trypsin treatment had approximately the same mobility and slope as Isozyme B2 and B3. This was confirmed by mixing the trypsinized Isozyme C with Isozymes B2 and B3 before running them on gels with the used five polyacrylamide concentrations.

TABLE I

RATE OF HYDROLYSIS OF AMINO ACID β-NAPHTHYLAMIDES EXPRESSED AS PER CENT OF MAXIMAL ACTIVITY

The B isozymes were assayed in the 102 000 × *g* supernatant of the M + L fraction, Isozyme C in the 102 000 × *g* pellet of the P fraction and Isozyme A in the S fraction.

β-Naphthylamide	Isozyme			
	B2 and B3	C after trypsin treatment	C	A
L-Alanyl	100	100	100	100
L-Leucyl	40.0	54.0	40.6	43.4
L-Isoleucyl	8.8	5.2	4.1	5.0
L-Arginyl	17.5	19.4	13.1	53.9
L-Lysyl	16.3	5.7	12.9	35.7
Glycyl	18.8	22.9	18.5	26.0
L-Prolyl	5.0	3.5	5.8	18.9
L-Cysteinyl	10.0	0.7	2.2	6.5

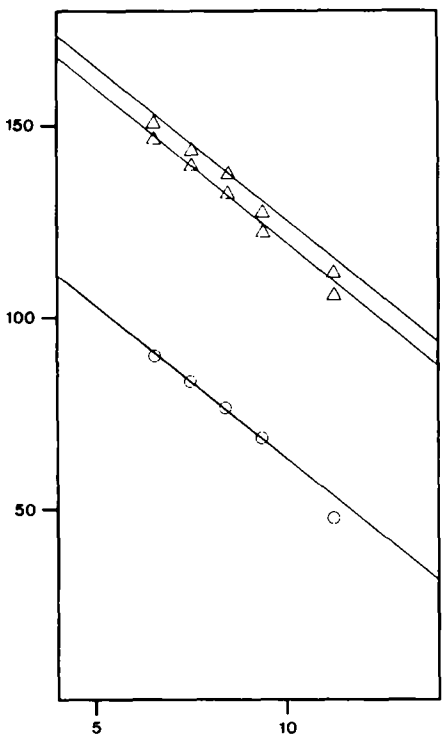

Fig. 8. Ferguson plot of the isozymes of amino acid naphthylamidase. Polyacrylamide gel concentration is plotted against relative electrophoretic mobility (R_m) expressed as 100 × log (R_m × 100). △—△, Isozyme C after treatment with trypsin, the solid lines represent the means of the values obtained after five runs with the M + L cell fraction (Isozyme B2 and B3). ○--○, Isozyme C. The points represents the means of two separate runs of the P fraction after solubilization with 1 % Triton X-100.

DISCUSSION

Conflicting results have been reported concerning the subcellular distribution of amino acid naphthylamidase. Lysosomal, microsomal and soluble naphthylamidases have been described. Sulvén and Lippi [22] found naphthylamidase activity in lysosomal subcellular vacuoles. Chayen et al. [23] have used amino acid naphthylamidases as markers for lysosomes in human synnovia.

Many authors have found a naphthylamidase in microsomal fractions. Rhodes et al. [24] found leucylnaphthylamidase in the microvillus membrane in hamster intestinal brush borders. Thomas and Kinne [25] assigned amino acid naphthylamidase to the surface of the microvillus matrix of brush border isolated from rat kidney cortex cells. Emmelot et al. [26] localized amino acid naphthylamidase on the brush border of rat liver cells on globular knobs. It was readily solubilized by papain, but not by trypsin.

Mahadevan and Tappel [27] found both lysosomal and microsomal naphthylamidases in rat liver and kidney. However, the enzymes from the two subcellular sources differed biochemically. These results have been extended by Kaulen et al. [28] in rat liver.

In this study, the formerly described microsomal enzyme has been shown to belong to plasma membranes contaminating the microsomal fraction of HeLa cells. As was earlier reported this enzyme is probably a structural membrane enzyme. The proposal that the microsomal and lysosomal isozymes are the same gene product [2] seems to be supported by the study of kinetic properties. Moreover, after treatment with trypsin, the plasma membrane-bound Isozyme C was readily solubilized and turned out to have the same electrophoretic mobility as the lysosomal Isozymes B2 and B3, while pertaining the same biochemical characteristics.

This raised the interesting question whether the microsomal Isozyme C is a precursor of the lysosomal Isozymes B2 and B3. This idea is supported by the kinetics of induction by glucocorticoids of these isozymes [6].

Lysosomal enzymes have been found to occur in microsomal fractions [7]. β-Glucuronidase is extensively studied and is located in the endoplasmic rectiulum besides the lysosomes [16]. Kato et al. [29] claimed the bulk microsomal enzyme to be a precursor of the lysosomal enzyme. This has been seriously questioned by Swank and Paigen [30], although the presence of a precursor of lysosomal β-glucuronidase residing in Golgi-derived material could not be ruled out.

Sloat and Allen [31] have described a membrane-bound and soluble lysosomal acid phosphatase in rat liver. The authors presented data, which supported a speculation that the membrane-bound activity resided in Golgi vesicles or young lysosomes and that conversion to the soluble isozyme occurred when the lysosomes matured. On the other hand a microsomal isozyme of acid phosphatase was demonstrated by Lin and Fishman [32] which was membrane bound and differed from a lysosomal isozyme. These isozymes were so dissimilar that the microsomal enzyme could not be a precursor of the lysosomal isozyme.

The lysosomal acid hydrolases are thought to be synthesized on the endoplasmic reticulum and transported to the Golgi apparatus for packing into primary lysosomes [7]. Goldstone and Koenig [33] have proposed that during this process the initially membrane-bound basic enzyme molecules are transformed to soluble acidic forms, by attachment of sialic acid. Apparently Isozyme C does not fit in this scheme. It appears to be located in the plasma membrane of cultured HeLa cells, while the possibility of a localization in the endoplasmic reticulum or the Golgi apparatus seems to be less probable. Treatment with neuraminidase did not transform Isozymes B2 and B3 into Isozyme C, which would be the consequence of Goldstone's and Koenig's proposal, although it is known that complete removal of sialic acid is not possible to achieve with neuraminidase [34]. According to our results, sialic acid seems already to be attached to Isozyme C, as after trypsin digestion of this isozyme the electrophoretic mobility of the resulting B-like isozymes was retarded by previous neuraminidase treatment of the P fraction. Then, the attachment of sialic acid is concommitant with and not the cause of the conversion to soluble, acidic isozymes. The spontaneous conversion at 37 °C but not at 0 °C is indicative of an endogenous proteolytic activity, the localization of which will be the subject of further studies.

Two possibilities must be considered for the interpretation of our results. (a) Isozyme C is a part of the plasma membrane which is infolded and integrated with the lysosomes during pinocytosis and formation of secondary lysosomes. Kaulen et al. [28] found plasma membrane-bound enzymes on the lysosomal membranes. However, these enzymes, including arylamidase, which probably is identical with amino

acid naphthylamidase [35], were too dissimilar to allow conclusions regarding a precursorship. (b) Isozymes B2 and B3 and Isozyme C are most probably the same gene product, but after synthesis they are transported to different parts of the cell. The membrane attachment of Isozyme C implies the addition of some trypsin-digestible residues on the enzyme molecule. β-Glucuronidase is an example of such a mechanism, where one and the same gene product is independently transported to two different subcellular organelles [30].

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