γ -Glutamylamine derivatives in isolated rat hepatocyte proteins

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Freshly isolated rat hepatocytes were found to contain a 3-fold higher level of putrescine than perfused liver. The bulk of this diamine was recovered in the acid-insoluble fraction of the cell. In order to determine the nature of the amine binding, the levels of γ -glutamylamine derivatives were measured. The method used involves exhaustive proteolytic digestion of the acid-insoluble fraction of hepatocytes, followed by ion-exchange chromatography. For N^1 -(γ -glutamyl)putrescine, a combined ion-exchange chromatographic and reverse-phase h.p.l.c. procedure was adopted. This allowed for the direct detection of less than 50 pmol of this derivative in enzymic hydrolysates. Several of the γ -glutamylamines reported previously [Beninati, Piacentini, Argento-Ceru', Russo-Caia & Autuori (1985) *Biochim. Biophys. Acta* **841**, 120–126] in the whole organ were found in the isolated liver cells. The elevated level of N^1 -(γ -glutamyl)putrescine and the absence of bis-(γ -glutamyl)spermine was noteworthy. The results suggest that, in rat hepatocytes, both polyamine-dependent post-translational modification of some proteins and cross-linking between proteins involving the glutamine and lysine residues occurs.

INTRODUCTION

Transglutaminases (EC 2.3.2.13) catalyse the post-translational modification of glutamine residues in a variety of proteins and peptides. In the presence of Ca^{2+} these enzymes promote an acyl-transfer reaction that results in isopeptide-bond formation between the glutamine and lysine residues of proteins and in the incorporation of small primary amines into protein via γ -glutamylamine covalent linkage (for reviews, see Folk & Finlayson, 1977; Folk, 1980; Lorand & Conrad, 1984). It has been demonstrated that aliphatic diamines and polyamines, e.g., putrescine, spermidine, spermine, can, through the action of transglutaminases, link both proteins and peptides via NN -bis-(γ -glutamyl)amine cross-bridges (Schrode & Folk, 1978; Folk, 1983). Although extracellular transglutaminases play significant physiological roles in fibrin polymerization and post-ejaculatory coagulation of rodent seminal plasma (Folk & Finlayson, 1977; Williams-Ashman, 1984), the precise function of the intracellular enzymes has yet to be clarified. Di- and poly-amines are ubiquitous components of living organisms. However, the physiological significance of the increased levels of free di- and poly-amines associated with normal and neoplastic growth is not fully understood. The discovery of the spermidine metabolite, hypusine, the product of a somewhat complex post-translational modification of eukaryotic initiation factor eIF-4D, provided the first example of a specific role played by a polyamine derivative in mammalian cells (Cooper *et al.*, 1982). It has been suggested that transglutaminase-catalysed covalent incorporation of naturally occurring di- and poly-amines occurs in proteins in both human plasma and amniotic fluid (Seale *et al.*, 1979; Chan *et al.*, 1979). Furthermore, the presence of radiolabelled γ -glutamyl di- and poly-amine derivatives has been reported in the proteolytic digests of acid-insoluble material from lymphocytes (Folk *et al.*, 1980),

of the marine gastropod *Aplysia californica* (Ambron & Kremzner, 1982), and of sea urchins during embryogenesis (Cariello *et al.*, 1984). γ -Glutamylhistamine has also been detected in the mast-cell line PT18 after activation (Fesus *et al.*, 1985).

We have shown recently that rat liver proteins contain covalently bound di- and poly-amines; the identification of endogenous N^1 -(γ -glutamyl)putrescine, N^1 -(γ -glutamyl)spermidine, N^1 -(γ -glutamyl)spermine and N^1N^{12} -bis-(γ -glutamyl)spermine in enzymic digests strengthens the argument that di- and poly-amines are natural transglutaminase substrates (Beninati *et al.*, 1985). In that study, however, the characterization of N^1 -(γ -glutamyl)putrescine could only be achieved indirectly by measuring the free diamine release after acid hydrolysis and by the action of γ -glutamylamine cyclo-transferase (Fink & Folk, 1983). In the present paper we report that the intracellular proteins of isolated hepatocytes contain covalently bound di- and poly-amines and ϵ -(γ -glutamyl)lysine cross-links. We also describe a procedure that allows the direct determination of N^1 -(γ -glutamyl)putrescine in proteolytic digests.

EXPERIMENTAL

Materials

ϵ -(γ -Glutamyl)lysine, N^1 -(γ -glutamyl)putrescine, N^1 -(γ -glutamyl)spermidine, N^8 -(γ -glutamyl)spermidine, N^1 -(γ -glutamyl)spermine and N^1N^{12} -bis-(γ -glutamyl)spermine were generously provided by J. E. Folk (National Institutes of Health, Bethesda, MD, U.S.A.). Pronase, collagenase, aminopeptidase M, carboxypeptidases A, B and Y were obtained from Boehringer, Mannheim, Germany. *o*-Phthalaldehyde (OPA), dansyl chloride, putrescine, spermidine, spermine and histamine were from Sigma. Usual laboratory chemicals were purchased from Merck (Darmstadt, Germany).

Abbreviations used: OPA, *o*-phthalaldehyde; PBS, phosphate-buffered saline (g/litre: NaCl, 8.0; KCl, 0.2; NaHPO₄, 0.2; KH₂PO₄, 0.2; pH 7.5).

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Table 1. Total di- and poly-amines in isolated hepatocytes

Portions of hepatocyte suspensions containing 30×10^6 cells were processed. Assays of di- and poly-amines were performed by reverse-phase h.p.l.c. as outlined in the Experimental section. The values in parentheses represent the data from perfused rat liver homogenate previously reported (Beninati *et al.*, 1985). Values are means $\pm$ S.D. for five determinations.

Di- or poly-amine	Total (nmol/mg of protein)	Acid-soluble (% of total)	Acid-insoluble (% of total)
Putrescine	0.3 ± 0.02 (0.1)	16 (35)	85 (58)
Spermidine	2.2 ± 0.30 (2.8)	95 (80)	5 (24)
Spermine	4.2 ± 0.20 (3.7)	95 (90)	3 (15)

Isolation of hepatocytes

Male Wistar albino rats weighing 200 g were used. Hepatocytes were isolated by using the collagenase perfusion technique described by Moldeus *et al.* (1978). This method yields consistently morphologically and biochemically intact cells, of which more than 95% excluded Trypan Blue (Autuori *et al.*, 1979).

Proteolytic digestion of cellular proteins

About 10^8 freshly isolated hepatocytes were washed three times in 30 ml of PBS. The cells were centrifuged, resuspended in 3 ml of PBS and lysed by treatment with cold trichloroacetic acid to a final concentration of 10% (w/v). The acid-insoluble material was collected after centrifugation at 3000 rev./min ($r_{av.} = 5.8$ cm) for 10 min and washed twice in 10 ml of 5% (w/v) trichloroacetic acid. Aliquots of the acid-insoluble material (in distilled water) were subjected to enzymic digestion as described previously (Beninati *et al.*, 1985).

Determination of γ -glutamylamine derivatives

Aliquots of proteolytic digests were analysed by automated ionic-exchange chromatography on a LKB Alpha Plus 4151 amino acid analyser equipped with a column (4.5 mm $\times$ 80 mm) packed with Ultrapac 8 resin (Na^+ form) using for elution the series of buffers previously reported (Folk *et al.*, 1980). The detection of the eluted compounds was performed by fluorescence from post-column OPA derivatization, using a Shimadzu RF-530 spectrofluorimeter. Collection of column effluent for external analyses was made directly from the column without mixing with fluorescent reagent.

Determination of N^1 -(γ -glutamyl)putrescine

N^1 -(γ -Glutamyl)putrescine is not completely separated from amino acids by ion-exchange-chromatographic methods (Folk *et al.*, 1980). Therefore further separation and detection of this compound was carried out by an h.p.l.c. procedure using precolumn derivatization by OPA. The OPA derivatives were obtained by incubating portions of sample (50–100 μ l) for 5 min at room temperature with 300–600 μ l of an assay mixture containing 200 mM-borate buffer, pH 10.4, 100 mM-OPA, 50% (v/v) methanol and 2% (v/v) β -mercaptoethanol. These samples were separated on an Altex Ultrasphere

ODS 5 μ m (4.6 mm $\times$ 150 mm) column (Beckman, Berkeley, CA, U.S.A.), using a linear gradient of acetonitrile/20 mM-sodium acetate buffer, pH 5.6 (1:4, v/v), and acetonitrile/20 mM-sodium acetate buffer, pH 5.6 (19:1) (elution time 20 min; flow rate 1.5 ml/min). A Beckman instrument equipped with two 114 M pumps, a data system 450/controller and a Shimadzu RF-530 spectrofluorimeter was used. This procedure was not suitable for resolving small quantities of N^1 -(γ -glutamyl)putrescine when there was a large amount of leucine in the sample. In order to overcome this limitation, the fraction corresponding to lysine, which also contains the putrescine derivatives, was collected from the ion-exchange-chromatographic-column effluent. This fraction does not contain leucine and other contaminating amino acids, and was suitable for analysis by the h.p.l.c. procedure described above.

Determination of ϵ -(γ -glutamyl)lysine

The analysis of ϵ -(γ -glutamyl)lysine was performed on portions of enzymic digest (50–100 μ l) by the reverse-phase h.p.l.c. procedure described by Griffin *et al.* (1982).

Assay of the di- and poly-amines

H.p.l.c. determination of di- and poly-amines was performed as described by Piacentini *et al.* (1986a), after acid hydrolysis of the samples in 6 M-HCl at 110 $^{\circ}$ C for 18 h.

Determination of protein

Protein determination was carried out by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

RESULTS

In a recent paper we reported that there are large amounts of endogenous di- and poly-amines covalently bound to protein in rat liver homogenates (Beninati *et al.*, 1985). The nature of this covalent binding was revealed through the detection of γ -glutamyl derivatives of these amines after exhaustive proteolytic digestion of the acid-insoluble fraction. The presence of these compounds and/or ϵ -(γ -glutamyl)lysine in biological material is evidence for transglutaminase activity *in vivo*.

To investigate the cellular localization of proteins post-translationally modified by di- and poly-amine incorporation, we have extended the previous study to hepatocytes. The hepatocytes utilized in the present study were intact and showed the morphological characteristics of their homologues *in vivo*. No contaminating cell types were identified in the preparation. Table 1 shows the total amounts of di- and poly-amines extracted from freshly isolated hepatocytes and their relative distribution in the acid-soluble and acid-insoluble fractions. Freshly isolated hepatocytes were found to contain a 3-fold higher level of putrescine than perfused liver homogenates. This higher concentration of putrescine was present mainly in the acid-insoluble fraction; the levels in the soluble fraction remained essentially unchanged. A decrease of 6- and 4-fold in acid-insoluble spermidine and spermine respectively was also seen. Again, little change was noted in the levels of polyamines isolated from acid-soluble fractions. To determine whether the hepatocyte acid-insoluble amines reported in

Fig. 1. Ion-exchange chromatographic separation of free di- and polyamines and their γ -glutamyl derivatives

The chromatographic conditions were those described by Folk *et al.* (1980). (a) Standard mixture of di- and polyamines and their γ -glutamyl derivatives. (b) Proteolytic digest of the acid-insoluble material from trichloroacetic acid-treated freshly isolated rat hepatocytes. The large band that was eluted between the beginning of the chromatogram and 40 min contains ammonia and free amino acids released by proteolytic digestion. 1, N^1 -(γ -glutamyl)putrescine; 2, N^1N^{12} -bis-(γ -glutamyl)spermine; 3, N^1 -(γ -glutamyl)spermidine; 4, N^8 -(γ -glutamyl)spermidine; 5, putrescine; 6, histamine; 7, N^1 -(γ -glutamyl)spermine; 8, spermidine; 9, spermine.

Table 1 were bound through γ -glutamyl linkages, we conducted a search for γ -glutamylamines in enzymic digests of this fraction in a manner similar to that performed for their identification in liver homogenates. In brief, this procedure involves exhaustive digestion of the cell homogenates with a combination of endo- and exo-peptidases having broad specificity. This releases the γ -glutamyl polyamine derivatives without modification of their chemical structures (Folk, 1983). Fig. 1 compares the ion-exchange chromatographic separation of a standard mixture of di- and polyamines and their γ -glutamyl derivatives (Fig. 1a) with a proteinase digest of the acid-insoluble fraction from rat hepatocytes (Fig. 1b). The digest displayed peaks corresponding to N^1 -(γ -glutamyl)spermidine and N^1 -(γ -glutamyl)spermine and also to free putrescine, histamine, spermidine and spermine, which were present even though the acid-

Fig. 2. H.p.l.c. analysis of N^1 -(γ -glutamyl)putrescine

The chromatographic conditions are reported in the Experimental section. (a) Standard mixture of 100 pmol of N^1 -(γ -glutamyl)putrescine (1) and 200 pmol of L-lysine (2). (b) Standard mixture of 300 pmol of free amino acids and about 20 pmol of N^1 -(γ -glutamyl)putrescine. (c) Analysis by h.p.l.c. of the ion-exchange chromatographic fraction corresponding to the retention time of N^1 -(γ -glutamyl)putrescine and lysine, obtained from the digest of rat hepatocyte acid-insoluble material.

insoluble fraction was washed thoroughly. The large broad band eluted in the first part of the digest chromatogram (Fig. 1b) contains small peptides, free amino acids and NH_3 . These materials prevent direct identification of N^1 -(γ -glutamyl)putrescine (see standard chromatogram). In order to measure this γ -glutamyl diamine we performed ion-exchange chromatography

Table 2. Free di- and poly-amines and their γ -glutamyl derivatives in the acid-insoluble fraction from rat hepatocytes

Portions of acid-insoluble fraction corresponding to about 100 mg of protein were digested. N^1 -(γ -Glutamyl)-putrescine determination was accomplished by the methods described in the Experimental section. Data are means $\pm$ S.D. for six determinations.

Compound	Concn. (pmol/ mg of protein)	Percentage of total
Putrescine	30.9 $\pm$ 17.3	8.8
N^1 -(γ -Glutamyl)putrescine	190.9 $\pm$ 28.2	54.8
Spermidine	14.5 $\pm$ 5.5	4.2
N^1 -(γ -Glutamyl)spermidine	55.5 $\pm$ 9.1	15.9
N^8 -(γ -Glutamyl)spermidine	Trace	—
Spermine	24.5 $\pm$ 14.5	7.0
N^1 -(γ -Glutamyl)spermine	32.1 $\pm$ 14.5	9.2

Table 3. Release of free di- and poly-amines from their γ -glutamyl derivatives by acid hydrolysis

γ -Glutamyl di- and poly-amines, obtained by proteolytic digestion of the acid-insoluble material from rat hepatocytes, corresponding to about 15 mg of protein, were separated by ion-exchange chromatography. N^1 -(γ -Glutamyl)putrescine was detected by the procedure described in the Experimental section. The chromatographic fractions corresponding to the individual γ -glutamyl derivatives were hydrolysed with an equal volume of 12 M-HCl by heating at 110 °C for 16 h. Portions of the samples were analysed to determine free di- and polyamines as described in the Experimental section.

Compound	Amount (pmol):	
	Before hydrolysis	After hydrolysis
N^1 -(γ -Glutamyl)putrescine	2200	—
N^1 -(γ -Glutamyl)spermidine	660	—
N^1 -(γ -Glutamyl)spermine	390	—
Putrescine	—	1800
Spermidine	—	460
Spermine	—	280

without mixing the effluent with the detection reagent, OPA, and collected the fractions that were eluted at the position of N^1 -(γ -glutamyl)putrescine. These fractions were suitable for h.p.l.c. analysis as outlined in the Experimental section and shown in Fig. 2. Fig. 2(a) shows the chromatographic analysis of a standard mixture containing 100 pmol of N^1 -(γ -glutamyl)-putrescine and 200 pmol of L-lysine. Since the quantity of amino acids in a proteolytic digest is considerably greater than that of the putrescine derivative, a standard mixture with an amino acids/ N^1 -(γ -glutamyl)putrescine ratio comparable with that of a digest was analysed (Fig. 2b). However, it was evident from this procedure that the resolution of small quantities of N^1 -(γ -glutamyl)-putrescine was impeded by the high leucine concen-

trations. The procedure outlined previously makes it possible to collect the fractions corresponding to L-lysine from an amino acid analyser. These fractions also contain mono-glutamyl derivative of putrescine, but not leucine, and are therefore suitable for analysis by h.p.l.c., since putrescine derivative separates well from lysine (Fig. 2c).

Table 2 lists the amounts and relative percentages of free di- and poly-amines and their γ -glutamyl derivatives found in the proteolytic digest of the acid-insoluble fraction of isolated rat liver hepatocytes. N^1 -(γ -Glutamyl)putrescine was by far the most prevalent amine derivative in the acid-insoluble fraction. The amount of this derivative was much higher in the isolated cells than in rat liver homogenate (Beninati *et al.*, 1985), suggesting a greater transglutaminase activity in hepatocytes than in whole liver. This may be a result of increased transglutaminase activity caused by proteinase treatment of the liver. Indeed, the hepatocyte isolation procedure utilizes collagenase, which is a potent activator of transglutaminase (Chung *et al.*, 1987). The amount of N^1 -(γ -glutamyl)spermidine represented about 16 % of the amines present in the acid-insoluble material, whereas the N^1 -(γ -glutamyl)spermine accounted for about 9 % (the amounts of these compounds were 10-fold lower than those found in rat liver homogenates). Bis-(γ -glutamyl)derivatives were not observed. The identity of the peaks that were eluted in the position of the γ -glutamylamines reported in Fig. 1(b) has been confirmed. In each case the release of free amine was observed after acid hydrolysis of the ion-exchange-chromatography fractions (Table 3).

About 5–6 nmol of ϵ -(γ -glutamyl)lysine/mg of protein were found in the digest of the acid-insoluble fraction of the freshly isolated hepatocytes. The identification of this compound was achieved by the detection of L-glutamine and L-lysine upon acid hydrolysis (results not shown).

DISCUSSION

It is well known that liver cells contain transglutaminases, the main form of which is the well characterized soluble 'tissue transglutaminase' (Folk, 1980). Recently, it was reported that rat liver also contains transglutaminase activity that is associated with the particulate fraction of the cells and mainly localized in the plasma membrane (Slife *et al.*, 1985). However, the natural substrates for these enzymes and their intracellular function are as yet unknown. In the present study we have identified di- and poly-amines covalently linked to proteins via a γ -glutamyl linkage and ϵ -(γ -glutamyl)lysine cross-links in freshly isolated hepatocytes. This finding suggests that intracellular transglutaminase activity has been expressed in these cells. Although these observations do not address the question of the intracellular role of transglutaminases, it is the first report documenting two types of γ -glutamyl derivatives in an essentially homogenous population of mammalian cells, both of which may be transglutaminase products. The present report complements and extends our earlier studies on rat liver (Beninati *et al.*, 1985). We provide evidence here for the presence in hepatocytes of several of the γ -glutamylamine derivatives identified previously in whole liver.

The high levels of N^1 -(γ -glutamyl)putrescine found in the hepatocytes (which represent the main cell type in

liver) suggests a preferential localization of the diamine derivative in these cells. In contrast, the 10-fold decrease of both N^1 -(γ -glutamyl)spermidine and N^1 -(γ -glutamyl)spermine strengthens the possibility of their wider distribution in the liver. Certainly, the identification in human plasma of fibronectin as a polyamine-binding protein (Mosher, 1976) and the reports on several peptides bearing polyamine residues (Seale *et al.*, 1979; Roch *et al.*, 1984) support this hypothesis. Bis-(γ -glutamyl)spermine was identified in whole liver homogenates, but was absent from the isolated hepatocytes. Although the basis of this difference is not yet known, it may be related to the presence of bis-(γ -glutamyl)spermine in apolipoprotein B isolated from the lipoprotein fraction of circulating human plasma (E. T. Cocuzzi, M. Piacentini, S. Beninati & S. I. Chung, unpublished work). These data, together with previous reports of the synthesis of γ -glutamylamine derivatives in stimulated lymphocytes (Folk *et al.*, 1980), in PT18 mast cells (Fesus *et al.*, 1985), in Chinese-hamster ovary cells (Beninati *et al.*, 1986) and in mouse epidermal cells (Piacentini *et al.*, 1986b) suggest that covalently bound di- and poly-amines may be ubiquitous in mammalian cells. The detection of significant amounts of γ -glutamyl di- and poly-amine derivatives and ϵ -(γ -glutamyl)lysine cross-links in isolated hepatocytes raises new and interesting questions about the regulation and the function of transglutaminase activity *in vivo*. Future studies on this subject should clarify whether the soluble and the particulate forms of transglutaminase reported in liver (Slife *et al.*, 1985) have different substrate specificity. Moreover, it would also be interesting to determine whether the post-translational modification of proteins involving di- and poly-amine incorporation is directed to different cellular proteins and/or to the same protein already bearing ϵ -(γ -glutamyl)lysine cross-links. In addition to the structural role served by the establishment of cross-links, transglutaminase-mediated post-translational modification of proteins might play a regulatory step in enzyme activity. The presence in isolated hepatocytes of only mono-(γ -glutamyl) di- and poly-amine derivatives seems to strengthen this hypothesis.

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