Formation of *N*^ε-(γ -glutamyl)-lysine isodipeptide in Chinese-hamster ovary cells

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N^ε-(γ -Glutamyl)-lysine isodipeptide was detected in a protein-free fraction of Chinese-hamster ovary cells and their culture fluid by using radioactive lysine as a tracer. The identity of the isodipeptide was established by its separation on ion-exchange chromatography, analysis by h.p.l.c. after derivatization, recovery of lysine after acidic hydrolysis or after cleavage by a specific enzyme, namely γ -glutamylamine cyclotransferase. The amount of isodipeptide was raised (460 pmol/10⁷ cells and 61 pmol/ml of culture fluid were observed as highest values) as the cell density increased. Effects of inhibitors of intracellular protein degradation have shown that the isodipeptide derives from cross-linking *N*^ε-(γ -glutamyl)-lysine bonds formed by tissue transglutaminase. Estimated half-life values of cross-linked proteins were about 3 h. γ -Glutamylamine cyclotransferase, which may split the isodipeptide formed during the continuous turnover of cross-linked proteins, was also found in Chinese-hamster ovary cells. Isodipeptide may have been accumulated when either its generated amount is beyond the capacity of γ -glutamylamine cyclotransferase or it is generated in cell compartments where this enzyme is not present.

INTRODUCTION

The *N*^ε-(γ -glutamyl)-lysine bond is primarily formed as a result of enzymic cross-linking of proteins by extra- and intra-cellular transglutaminases [1,2]. Cross-linking of fibrin is catalysed by the plasma transglutaminase Factor XIII at the last stage of blood coagulation [3]. Cornified envelopes are formed by keratinocyte transglutaminase during the terminal differentiation of epidermal cells [4,5]. Tissue transglutaminase, a product of a third transglutaminase gene [6], is a Ca²⁺-dependent intracellular enzyme which has been suspected to participate in a wide range of cellular functions, including regulation of cell proliferation [7], differentiation [8], receptor-mediated endocytosis [9], secretion of hormones [10], aging [11] and programmed cell death [12]. Since identification of substrate proteins *in situ* and demonstration of their cross-linkage have not been carried out so far, the function of the enzyme is still obscure.

One way to learn more about transglutaminase-catalysed protein cross-linking in cells is to study the kinetics of the formation and degradation of the *N*^ε-(γ -glutamyl)-lysine bond. It has been shown that Chinese-hamster ovary cells have a substantial level of tissue transglutaminase, which forms high-molecular-mass protein polymers in intact cells [13–15]. Here we report that the isodipeptide *N*^ε-(γ -glutamyl)-lysine is accumulated in these cells and their culture fluid as a result of continuous proteolytic breakdown of cross-linked proteins. γ -Glutamylamine cyclotransferase, which specifically cleaves the isodipeptide [16], is also present in these cells.

MATERIALS AND METHODS

Materials

[³H]Lysine (97 Ci/mmol) was purchased from New England Nuclear. γ -Glutamyl dansylcadaverin, γ -glutamylputrescine, *N*^ε-(γ -glutamyl)-lysine and ³H-labelled *N*^ε-(γ -glutamyl)-lysine (1.23 Ci/mmol; produced by using [³H]lysine) were synthesized by conventional methods [17]. Leupeptin and chymostatin were obtained

from Sigma, and proteolytic enzymes (Pronase, leucine aminopeptidase, carboxypeptidases A and B) were from Boehringer Mannheim.

Cell culture and fractions

Chinese-hamster ovary cells were cultured in Falcon tissue-culture dishes at 37 °C under CO₂/air (1:19) in α -modified Eagle medium supplemented with 10% (v/v) fetal-calf serum, 2 mM-glutamine, penicillin (50 i.u./ml) streptomycin (50 μ g/ml) and, in most of the experiments, with [³H]lysine (final sp. radioactivity 37 μ Ci/ μ mol). Cells were seeded at 5 $\times$ 10⁵ per 75 cm² dish. At various time intervals the culture fluid was collected and cells were washed three times in cold iso-osmotic buffer [25 mM-Tris/HCl (pH 7.5)/145 mM-NaCl/0.5 mM-EDTA], scraped from the dishes with a rubber policeman, centrifuged at 800 *g* for 5 min, then disrupted by freezing and thawing three times and by a brief sonication in the presence of a low-ionic-strength buffer [10 mM-Tris/HCl (pH 7.5)/5 mM-KCl/1 mM-EDTA/0.5 mM-phenylmethanesulphonyl fluoride/0.2 mM-leupeptin]. Portions of cell lysates were centrifuged at 100 000 *g* for 1 h and the supernatant was collected. This supernatant, and the culture fluid, were deproteinized at +4 °C by either centrifugation using a Centrifree partition device (Amicon) or by trichloroacetic acid (10% final concn.) precipitation. Protein-free fractions were used for further analysis. Portions of cell lysates were precipitated by trichloroacetic acid (protein fractions of cells), and the precipitates were processed for proteolytic digestion as described in detail elsewhere [18,19]. After extensive digestion with Pronase, leucine aminopeptidase and carboxypeptidases A and B, samples were precipitated again with trichloroacetic acid, then the supernatant was treated with diethyl ether and processed for chromatographic analysis.

Separation and identification of *N*^ε-(γ -glutamyl)-lysine

Protein-free cell lysates and culture fluids, as well as proteolytic digests of cell proteins, were passed through

an AG-50W-X8 resin (Bio-Rad Laboratories) cation-exchanger column (NH_4^+ form) to remove lysine. Samples were concentrated by freeze-drying, then analysed by an ion-exchange chromatography system [18] using a column (0.4 cm $\times$ 8 cm) of DC6A resin (Dionex), the buffer system described in the legend of Fig. 1 (below) and elution at 66 °C at a flow rate of 38 ml/h. Fractions were collected to determine their radioactivity. The radioactive material eluted at the position of N^ϵ -(γ -glutamyl)-lysine was either acid-hydrolysed (6 M-HCl at 110 °C for 14 h) or incubated in the presence of 25 μg of γ -glutamylamine cyclotransferase/ml (purified as described by Fink *et al.* [16]) at 37 °C for 3 h. After these treatments samples were re-run on the same column to determine the retention time of the radioactive material. In some cases the fraction eluted at the position of N^ϵ -(γ -glutamyl)-lysine was derivatized with *o*-phthalaldehyde and the fluorescent products were separated on a 3.9 mm $\times$ 30 cm μ Bondapak C_{18} column (Waters Associates) by h.p.l.c. using an elution system [linear gradient from 10 mM-sodium acetate to 10 mM-sodium acetate/acetonitrile (1:4, v/v)] described for the detection of γ -glutamylpolyamines and N^ϵ -(γ -glutamyl)-lysine [20]. The radioactivity was determined in the fraction collected at the position of the derivatized synthetic isodipeptide. In another series of analyses the protein-free fractions were passed through the AG-50W-X8 column to remove lysine, then desalted on a silica h.p.l.c. column using water as eluent. The fraction collected at the elution position of N^ϵ -(γ -glutamyl)-lysine was derivatized with phenylisothiocyanate [21], then run on the above-specified C_{18} column to determine radioactivity in the fraction eluted at the position of derivatized pure N^ϵ -(γ -glutamyl)-lysine.

Measurement of γ -glutamylamine cyclotransferase activity

Cells were disrupted in a buffer containing 20 mM-sodium phosphate, pH 7.5, 1 mM-EDTA, 0.5 mM-phenylmethanesulphonyl fluoride and 0.2 mM-leupeptin. Measurement of enzyme activity was carried out according to a published procedure, with N^ϵ -(γ -glutamyl)-lysine as a substrate [16]. The concentration of protein in the cell lysate was determined by the Bradford method [22].

RESULTS

Detection of N^ϵ -(γ -glutamyl)-lysine isodipeptide in a protein-free fraction of Chinese-hamster ovary cells

Cells were grown in a medium which contained the usual concentration of lysine (0.272 mmol/l) and a trace amount of [^3H]lysine (final sp. radioactivity 37 $\mu\text{Ci}/\mu\text{mol}$). Cells of pre-confluent cultures (approx. 1×10^7 cells/dish) were collected and their protein-free fractions, prepared by using either trichloroacetic acid precipitation or molecular filtration, were analysed in an amino acid analyser after the removal of lysine by a cation-exchange column. When fractions were monitored, a radioactive peak emerged at a position corresponding to that of synthetic radiolabelled N^ϵ -(γ -glutamyl)-lysine (Fig. 1a). Identity of the radioactive material was established by four independent criteria: (1) acidic hydrolysis of the radioactive fraction eluted at the position of the isodipeptide resulted in the appearance of lysine in equimolar quantity (Fig. 1b); (2) pretreatment of the sample before analysis using γ -glutamylamine

Fig. 1. Detection of N^ϵ -(γ -glutamyl)-lysine isodipeptide in a protein-free fraction of Chinese-hamster ovary cells by ion-exchange chromatography

Chromatography was performed as described by Folk [17], the following elution program being employed: sodium citrate buffer (Na^+ 0.2 mol/litre), pH 3.31, 12 min; sodium citrate buffer (Na^+ 0.2 mol/litre), pH 4.31, 19 min; sodium citrate buffer (Na^+ 0.6 mol/litre), pH 5.8, 7 min; sodium citrate buffer (Na^+ 0.2 mol/litre) pH 3.31, 17 min. Fractions were collected for 1.5 min. (a) Distribution of radioactivity in a protein-free fraction prepared by trichloroacetic acid precipitation of cell proteins. (b) Fraction 8 of run (a) was either acid-hydrolysed (○) or was neutralized to pH 7.5, then incubated in the presence of γ -glutamylamine cyclotransferase and run again on the analyser (■). Cells were cultured in the presence of [^3H]lysine and their protein-free fractions were processed for chromatography as described in the Materials and methods section. The analysed sample corresponds to 0.3×10^7 cells. 'GluLys' and 'Lys' show the exact elution positions of standard N^ϵ -(γ -glutamyl)-lysine and lysine respectively.

cyclotransferase, an enzyme which specifically cleaves N^ϵ -(γ -glutamyl)-lysine [16], resulted in the almost complete disappearance of the radioactive peak in the isodipeptide region with the concomitant appearance of radioactivity in the lysine region (Fig. 1b); (3) the material eluted at the position of the isodipeptide was derivatized with *o*-phthalaldehyde, then analysed by h.p.l.c. The radioactive peak appeared at the position of *o*-phthalaldehyde-derivatized pure N^ϵ -(γ -glutamyl)-lysine (results not shown); (4) when molecules in the protein-free fraction of cells were derivatized with phenyl isothiocyanate and subjected to h.p.l.c., a radioactive peak was detected at the position of phenyl isothiocyanate-derivatized synthetic N^ϵ -(γ -glutamyl)-lysine (results not shown).

On the basis of the specific radioactivity of lysine, the

amount of isodi peptide could be calculated: a value of 188–238 pmol/10⁷ cells (10⁷ cells correspond to approx. 1 mg of protein) was obtained in a series ($n = 8$) of pre-confluent cultures, that is, 48 h after seeding 5×10^5 cells into a 75 cm² tissue-culture dish. A detectable concentration, in the range 35–48 pmol/ml, was found in the culture fluids as well. There was no difference in the results when the two methods of sample deproteinization, i.e. precipitation and molecular filtration, were compared using identical samples. All calculations were based on the initial specific radioactivity of lysine in the media. In a series of preliminary experiments, no significant difference was found, at least from the 20 h time point and onwards, when the lysine content of protein-free cell fractions and of samples obtained by acid hydrolysis was either determined directly by derivatization with phenyl isothiocyanate or was calculated on the basis of lysine specific radioactivity (results not shown). It has been assumed throughout the experiments that intracellular pools of unlabelled lysine and/or transport of labelled lysine does not vary during growth and confluency.

Control experiments were carried out to investigate probable factors which may affect the results obtained above. Formation of isodi peptide was not observed when media with radioactive lysine was kept in the incubator for 24 h. Likewise, radioactive isodi peptide was not found when cell lysate (prepared by disruption of cells in low-ionic-strength buffer) was incubated for various time intervals at 37 °C in the presence of [³H]lysine. In a separate experiment, radioactive isodi peptide was added to pre-confluent cells: after 24 h incubation it was completely recovered as unchanged N^ε-(γ-glutamyl)-lysine, showing that the isodi peptide once formed and released is not taken up by the cells and not modified in the culture fluid.

N^ε-(γ-Glutamyl)-lysine in proteins and protein-free fractions of cells and culture fluid in different phases of cell growth

N^ε-(γ-Glutamyl)-lysine was found in proteins of cells proliferating rapidly after the initiation of their growth (Fig. 2). Its amount in a culture dish increased continuously while the number of cells increased, showing maximum values when cells reached confluency. The isodi peptide was detected in cells as well as in the media at the early phase of cell growth, and increased at higher cell densities, with maximum values (460 pmol/10⁷ cells and 61 pmol/ml of culture fluid) at confluency.

Inhibition of isodi peptide formation by inhibitors of intracellular protein degradation

The presence of methylamine, an inhibitor of lysosomal degradation of proteins [23], could almost completely prevent the appearance of N^ε-(γ-glutamyl)-lysine isodi peptide in cells and culture fluid (Table 1). At the same time the concentration of protein-bound isodi peptide was substantially increased. Leupeptin, a well-known peptide inhibitor of intracellular protein degradation [24] was also effective in preventing the formation of isodi peptide. The increase of protein-bound isodi peptide concentration in leupeptin-treated cells was lower than in methylamine-treated ones. Chymostatin, which is considered mostly as an inhibitor of non-lysosomal intracellular protein degradation [24], did not influence the formation of isodi peptide as effectively as did methylamine or leupeptin (Table 1).

Fig. 2. Changes of cell number and the amount of N^ε-(γ-glutamyl)-lysine isodi peptide in protein-free fractions of cultured Chinese-hamster ovary cells

▲, Isodi peptide in cells; ■, isodi peptide in media. N^ε-(γ-glutamyl)-lysine values and cell numbers (○) correspond to amounts found in one 75 cm² culture dish.

Table 1. Effect of inhibitors of intracellular protein degradation on the formation of isodi peptide

Compounds were added 24 h after seeding 5×10^5 cells and were present for 12 or 24 h. Results show typical values obtained in several experiments; each corresponds to the amount found in one 75 cm² culture dish.

Inhibitor	Time in culture (h)	N ^ε -(γ-glutamyl)-lysine (pmol) in:		
		Protein	Protein-free fraction	
			Cells	Medium
None	12	2634	68	108
	24	3267	228	415
Methylamine (20 mM)	12	3850	2	0
	24	6345	35	16
Leupeptin (0.2 mM)	12	3350	6	0
	24	4638	18	40
Chymostatin (0.2 mM)	12	2980	26	34
	24	4124	87	167

Approximate turnover rate of proteins containing N^ε-(γ-glutamyl)-lysine

When the amounts of isodi peptide bond in cell proteins and in protein-free fractions (cells plus media) were

compared, the latter did not reach more than 30% of the total amount of *N*^ε-(γ -glutamyl)-lysine in the culture at any time point investigated. Consequently, either the turnover rate of protein(s) containing the cross-link is very low or there is an enzyme which continuously cleaves the released isodipeptide. To study the turnover rate, cells were grown in media with [³H]lysine. In some of the cultures the medium with [³H]lysine was replaced by a fresh one without labelled lysine. *N*^ε-(γ -Glutamyl)-lysine of proteins was determined in cells which were grown in media containing [³H]lysine only for a limited time, and the values were compared with those obtained in cells which were grown continuously in the presence of [³H]lysine. Calculations were carried out using the same [³H]lysine specific-radioactivity value throughout the experiment, namely the one which corresponds to the starting media. The change of medium was carried out at three time points. In all cases interrupting the supply of [³H]lysine resulted in a decrease of the amount of radioactive isopeptide in proteins (Fig. 3), but the response was delayed and the values did not reach levels close to zero. Metabolically labelled substrates of transglutaminase, which are eventually cross-linked even after a change of medium, are certainly present at the time of changing the culture fluid. Therefore the turnover rate of cross-linked proteins cannot be precisely determined. Keeping the limitation of the system in mind, approximate half-life values were calculated on the basis of data presented in Fig. 3. Proteins containing *N*^ε-(γ -glutamyl)-lysine were degraded with a half-life of about 3 h (considering the initial phase of their disappearance). The degradation could be prevented by addition of leupeptin to the cells (Fig. 3).

Fig. 3. Change of the amount of *N*^ε-(γ -glutamyl)-lysine (GluLys) bond in proteins of cells

▲, *N*^ε-(γ -Glutamyl)-lysine in the protein fraction of cells growing continuously in the presence of labelled lysine; △ (broken line), *N*^ε-(γ -glutamyl)-lysine in protein fraction of cells which were grown first with [³H]lysine, then, from the time points indicated by arrows, without it; □, samples were prepared as for △, except that leupeptin (0.2 mM) was added at the time of removal of [³H]lysine; ○, cell no. Values were calculated on the basis of the specific radioactivity of lysine in the media at the beginning of the experiment. All results correspond to amounts found in one 75 cm² culture dish.

γ -Glutamylamine cyclotransferase activity in Chinese-hamster ovary cells

The calculated turnover rate of cross-linked proteins should result in the accumulation of much higher amounts of isodipeptide in the culture than those observed in our experiments. Consequently, there must be an enzyme which can split the isodipeptide. Such an enzyme, called ' γ -glutamylamine cyclotransferase' was described previously [16]. Assaying cell extracts for the presence of this enzyme showed a pronounced activity in proliferating (52 nmol/h per mg of protein), pre-confluent (89 nmol/h per mg of protein) and confluent (76 nmol/h per mg of protein) cells. The velocity of cleavage of *N*^ε-(γ -glutamyl)-lysine could be inhibited by γ -glutamyl dansylcadaverine and γ -glutamylputrescine (results not shown), which are also good substrates of γ -glutamylamine cyclotransferase.

DISCUSSION

Although the existence *in vivo* and widespread occurrence of intermolecular *N*^ε-(γ -glutamyl)-lysine bonds have been demonstrated [25,26], the formation of protein-free isodipeptide in cells has not so far been demonstrated. It is known that the isopeptide bond is resistant to proteinase action [25]. Consequently, *N*^ε-(γ -glutamyl)-lysine isodipeptide should be formed during intracellular proteolytic degradation of cross-linked proteins. On the basis of this assumption we could find isodipeptide in Chinese-hamster ovary cells and their culture fluid. Combining the amounts in cells and media, approx. 1 nmol was usually found as a maximal amount in a 75 cm² culture dish where about 2×10^7 cells were present at maximum cell density. Calculating the concentration as a function of cell volume, it reaches the micromolar range. There is a single report showing that *N*^ε-(γ -glutamyl)-lysine isodipeptide is present in the blood plasma of the male winter flounder *Pseudopleuronectes americanus*, and its concentration varies seasonally, reaching highest values at the time of spawning [27].

Our results show that the source of isodipeptide is the *N*^ε-(γ -glutamyl)-lysine bond in proteins of cells. The obvious explanation of the presence of *N*^ε-(γ -glutamyl)-lysine in proteins is that they are cross-linked by a transglutaminase. The transglutaminase in Chinese-hamster ovary cells is of the tissue type, and its increased activity during high cell density was demonstrated previously [13,15]. Simultaneous occurrence of high transglutaminase activity, high concentration of *N*^ε-(γ -glutamyl)-lysine in proteins and high cell density of WI-38 human fibroblasts was also reported [7]. However, alternative routes for the formation of *N*^ε-(γ -glutamyl)-lysine in proteins have been also reported [28,29]. α_2 -Macroglobulin and complement factors 3b and 4 contain a highly reactive internal thioester bond which is either hydrolysed or forms a covalent bond with the ϵ -amino group of lysine side chains of other proteins after limited proteolysis. Such a thioester bond could not be detected in our system with radioactive methylamine, which can react with the thioester bond even when it is in the intact molecule [28,29].

There is no available data in the literature about the turnover rate of proteins cross-linked by tissue transglutaminase in cells. Intracellular proteins are degraded

through several intracellular pathways, resulting in half-lives ranging from minutes to days [30]. A significant degradation rate of cross-linked proteins is suggested from the results of the experiment in which proteinase inhibitors were added to our cultures: both prevention of the accumulation of isodipeptide and a substantial increase of N^ε-(γ-glutamyl)-lysine in intact proteins could be observed. Since a specific inhibitor of tissue transglutaminase is not available, it is not a simple task to determine the precise turnover rate of the proteins containing the isopeptide bond. After the removal of radioactive lysine, a pool of not cross-linked but labelled proteins is probably still available for transglutaminase as substrate(s). Further cross-linking of these proteins may result in an increase of the labelled bond first, leading to underestimation of the real turnover rate. Even without correcting for this component of the system, a quite high turnover rate can be observed. The breakdown may be estimated to proceed with a half-life of 3 h.

Substrates of tissue transglutaminase have not so far been identified in cells and tissues. High-molecular-mass polymers, into which low-molecular-mass amine substrates of transglutaminase can be incorporated, were found in Chinese-hamster ovary cells [14,15], mast cells [19], pancreatic β-cells [10] and liver [31,32]. Neither the relationship between their formation and a particular cell function nor their fate has been established. The difficulties encountered so far in the process of identification of transglutaminase substrates and functions may be partially explained by the fast turnover of cross-linked proteins revealed in the present paper.

The high disappearance rate of cross-linked proteins and the discrepancy between the expected and observed amount of the isodipeptide raises the possibility that an enzyme capable of reversing the transglutaminase-catalysed reaction in proteins may function in Chinese-hamster ovary cells. However, the existence of such an enzyme has not been demonstrated in mammalian cells. There is a preliminary report about an enzyme in leeches [33] which seems to reverse cross-linking of fibrin by splitting the N^ε-(γ-glutamyl)-lysine bond in the intact molecule. It is unlikely that cleavage of this bond occurs through the reversed action of transglutaminase, because this reaction is slow and inefficient [34]. Furthermore, our results clearly point to the involvement of proteolytic degradation in the breakdown of cross-linked proteins.

The presence of γ-glutamylamine cyclotransferase in Chinese-hamster ovary cells sufficiently explains the relatively low amount, as compared with values calculated on the basis of the degradation rate of cross-linked proteins, of isodipeptide in cells and the culture fluid. This enzyme specifically breaks bonds produced by transglutaminase when the modified proteins are degraded by proteinases and the γ-glutamyl derivatives are released from the polypeptide chain [16]. Our results provide support for the suggestion that transglutaminase-modified proteins in cells are degraded in such a manner that substrates for γ-glutamylamine cyclotransferase are rapidly and preferentially formed [35]; this idea has been particularly attractive considering that post-translationally modified proteins and those with abnormal structures are degraded rapidly in cells [36,37].

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