Activation of Transglutaminase and Production of Protein-bound γ-Glutamylhistamine in Stimulated Mouse Mast Cells*

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The identification of transglutaminase in the growth-factor-dependent mouse mast cell line PT18 was accomplished through its characteristic catalytic properties (specificity, calcium dependency, and inhibition by iodoacetamide); and by both immunoprecipitation and Western blot analysis using affinity purified antibody. The enzymatic activity in these cells increased in association with the release of histamine from the cells induced by an IgE-dependent mechanism or by exposure to the ionophores A23187 or Br-x537A. The increase in transglutaminase activity was paralleled by a marked increase in the level of protein-bound γ-glutamylhistamine, determined in radiolabeled form in mast cells that were either metabolically labeled with [3H]histidine or incubated with [3H]histamine before degranulation. The highest level of bound γ-glutamylhistamine was found in the immunologically stimulated cells. Enzymatic activity and the γ-glutamyl derivative were associated primarily with the cells, both before and after stimulation. Separation of γ-glutamylhistamine in a proteolytic digest of these cells was carried out using a combination of ion exchange chromatography and high performance liquid chromatography. The γ-glutamyl compound was identified and quantitated through the enzymatic production of histamine with the use of γ-glutamylamine cyclotransferase, an enzyme specific for the disassembly of γ-glutamylamines.

The transglutaminases catalyze a calcium-dependent acyltransfer reaction between peptide-bound glutamine residues and primary amines including the ε-amino group of lysine residues in appropriate peptides. The production of ε-(γ-glutamyl)lysine bonds between proteins is perhaps the most important in vivo function of the transglutaminases. Prominent examples include the covalent polymerization of fibrin, the formation of a vaginal plug postejaculation in rodents, and the production of a cornified envelope during terminal differentiation of keratinocytes (for reviews, see Folk and Finlayson, 1977; Folk, 1980; Thacher and Rice, 1985). Further, a variety of primary amines can serve as substrates for transglutaminases, this was considered to be an in vitro phenomenon. Interest in this area, however, led to the development of sensitive assay systems for transglutaminases (Lordan and Ong, 1986; Pincus and Waelsch, 1968; Folk and Chung, 1973; Gross et al., 1977). Recent studies have provided evidence for a possible physiological substrate function for primary amines, namely the polyamines prevalent in cells and body fluids. γ-Glutamyl derivatives of polyamines have been identified in proteolytic digests of mitogen-stimulated lymphocytes and clotted seminal plasma (Folk et al., 1980). In addition, indirect evidence for a possible physiological substrate function is thus given substantial support.

EXPERIMENTAL PROCEDURES

Materials—Br-x537A (provided by Dr. W. E. Scott, Hoffman-La Roche Inc., Nutley, NJ), A23187 (Calbiochem-Behring), Carboxypeptidase A, B, and Y (Sigma), Pronase and aminopeptidase M (Boehringer Mannheim), Protein ASepharose-4B (Pharmacia Fine Chemicals), and prelabeled molecular weight standards (Bethesda Research Laboratories) were obtained from the sources indicated. [ring 2,side chain-3-3H]Histidine (12.5 Ci/mmol), [ring methylenes-3H]histamine-2HCl (52 Ci/mmol), and [4,5-3H]leucine (52.9 Ci/mmol) were purchased from New England Nuclear. Succinylated β-casein and guinea pig liver transglutaminase were the gifts of Dr. S. I. Chung (National Institute of Dental Research, National Institutes of Health, Bethesda, MD). γ-Glutamylamine cyclotransferase was purified by the published procedure (Fink and Folk, 1983) and was of purity similar to that reported. Rabbit anti-transglutaminase antibodies were prepared by immunization of rabbits with purified human red blood cell transglutaminase. The anti-transglutaminase antibodies were purified from the globulin fraction of the immune serum by affinity chromatography on a column of guinea pig liver transglutaminase immobilized on cyanogen bromide-activated Sepharose. Details of the preparation, purification, and characterization of these antibodies are described elsewhere (Fesus and Arato, 1985).

Growth and Maintenance of PT18 Cells—The PT18 cell line was obtained as a subline of a parent murine cell line that was isolated from antigenically stimulated spleen cells (Pluznik et al., 1982). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂, 95% air, in RPMI 1640 medium (DUTCH modification, Flow Laboratories, McLean, VA) to which had been added L-glutamine (4 mM), 2-mercaptoethanol (50 μM), penicillin (50 units/ml), streptomycin...
was supplemented as described above. In some experiments the cells were grown for gentle agitation. The cells were next washed twice in the HEPES-buffered Tyrode's solution to remove unbound antibody. The release was increased to pH 7.4 (Barrett et al., 1983). For experiments involving an immunologic stimulus, 5-4 × 10⁷ cells/ml were then passively sensitized with hybridoma-derived mouse anti-ovalbumin IgG (a gift from Dr. B. P. Siragianian, National Institute of Dental Research, National Institutes of Health, Bethesda, MD) 1:50 dilution for 1 h at 37°C with gentle agitation. The cells were next washed twice in the HEPES-buffered Tyrode's solution to remove unbound antibody. The release experiments were carried out at 37°C according to the method of Atkinson et al. (1979) using ~1-2 × 10⁶ cells/reaction tube in a volume of 1 ml. (In the experiments when proteolytic digestion of cellular proteins followed stimulation, the number of cells was increased to 4-6 × 10⁶/tube in 2 ml). Passively sensitized cells were challenged with aggregated ovalbumin (10 μg/ml). Experiments involving nonimmunologic secretagogues (ionophore A23187, 10 μM; and ConA, 10 μg/ml) utilized cells which had been washed but not passively sensitized. Release was terminated at 10 min by the addition of an equal volume of ice-cold buffer or 20% trichloroacetic acid (the latter was used in the digestion experiments). The cells were immediately separated from the released histamine in the supernatant by centrifugation and 10 min (in fresh Tyrode's buffer to release residual histamine. Histamine in the cellular and supernatant fractions was assayed fluorimetrically (Shore et al., 1983), either manually or using a commercial autoanalyzer (Alpkem, Clackamas, OR).

**Assay of Transglutaminase Activity**—After incubation and stimulation to degranulate, ~1-2 × 10⁷ PT18 cells were centrifuged, suspended in 300 μl of TBS (Tris-buffered saline:20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.6), and disrupted by quickly freezing and thawing three times. Portions of the cell extract (100-200 μg of protein) were incubated at 37°C in a total volume of 200 μl of TBS containing 1 mM diithiothreitol, 4 μg of succinylated β-casein, 5 mM CaCl₂, 2 mM histamine (in excess of the amount contributed by the cells), 0.5 μM [³H]histamine, and 0.1 μM aminoguanidine (to inhibit histaminase) at pH 7.6. At 3- and 15-min intervals 80-μl aliquots were withdrawn and incubated in the presence of 5 μM trichloroacetic acid exhaustively washed with 5% trichloroacetic acid on GF/A Whatman filters. The protein-bound radioactivity was determined in Hydrofluor filters and the resulting precipitate was suspended in 0.2 M N-ethylmorpholine acetate buffer (pH 8.1) using a G-25 Sephadex column.

**RESULTS**

In order to explore the possibility that histamine serves as a transglutaminase substrate in normal cells in culture, we chose for study a pure cell population that contains large amounts of histamine and we examined the relationship between the transglutaminase levels and the histamine covalently attached to protein through amide linkage at the γ-carboxyl group of glutamic acid residues.

**Transglutaminase in PT18 Mouse Mast Cells**—The cell population employed here is a recently described mouse cell line, PT18, derived from spleen (Pluznik et al., 1982). This cell line requires growth factors for proliferation, contains histamine (~1 pg/cell), expresses Fc receptors, and has been shown to rapidly release histamine in response to immunologic as well as nonimmunologic stimuli (Barrett et al., 1984).

Using monospecific anti-transglutaminase antibody, a protein similar in molecular weight to guinea pig liver transglutaminase, was immunoprecipitated from metabolically labeled PT18 cells (Fig. 1A, lane 2). Addition of an excess amount of guinea pig transglutaminase to the cell extract neutralized the anti-transglutaminase antibody (Fig. 1A, lane 3). Western blot analysis of the PT18 cell extract following SDS-polyacrylamide gel electrophoresis revealed a single band of immunoreactive material of apparent molecular weight 77,000 (Fig. 1B). The antibody showed a strong and specific reaction with the transglutaminases of guinea pig liver (apparent = 92,000), mouse liver (apparent = 80,000), and human red blood cells (apparent M, = 92,000). The heterogeneous pattern of species differences for migration of the enzyme in SDS gels is in agreement with that reported previously (Murtaugh et al., 1983a).

The level of transglutaminase protein in PT18 cell extracts, measured by means of a recently developed ELISA technique (Pusas and Arato, 1980), was found to be 55 ± 14 (mean ±

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1 The abbreviations used are: ConA, concanavalin A; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay.
Tris-HCl, 150 mM NaCl, 1 mM EDTA (pH 7.5) (TBS), suspended in 3 ml of 50 mM Tris-HCl (pH 7.6) with 10 mM NaCl, 10 mM benzamidine (lanes 1, 2, and 3). The Protein A-immune complex precipitates were centrifuged at 1000 rpm for 5 min, washed 3 times with 10 ml of lysing buffer, and processed for SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) on 7.5% discontinuous slab gels. Radioactive bands were located by fluorography on Kodak X-OMAT AR film. B, the Western blot procedure was employed to detect unlabeled tissue transglutaminase in extracts. PT18 cells (2 × 10^6 in TBS) were lysed by sonication and directly denatured in sample buffer for electrophoresis. After fractionation by SDS electrophoresis the proteins were electroblotted onto nitrocellulose (Hawkes et al., 1982) and visualized using the Bio-Rad Immun-blot (GAR-HRP) assay kit. Affinity purified anti-transglutaminase antibody was used at a concentration of 2 μg/ml. Lane 1, human red blood cell transglutaminase (5 ng); lane 2, the prestained molecular weight standards, myosin heavy chain, phosphorylase b, bovine serum albumin, chymotrypsin, lactoglobulin, respectively (molecular weights are given in kilodaltons; lanes 3 and 7, Chinese hamster ovary cell extract (50 μg of protein); lane 4, cell extract (100 μg) prepared from PT18 cells grown in the presence of 1 mM sodium butyrate for four days; lane 5, PT18 cell extract (100 μg); lane 6, PT18 cell extract (100 μg) immunoprecipitated by anti-transglutaminase antibody before electrophoresis; lane 8, purified guinea pig liver transglutaminase (50 ng).

S.E., n = 4) ng/mg cell protein based on the use of the guinea pig liver enzyme as a standard. The level of enzyme protein (77 ± 12 ng/mg protein) found after growth of the PT18 cells for 5 days in the presence of sodium butyrate was not significantly different, although sodium butyrate is known to stimulate transglutaminase synthesis in fibroblasts (Birckbichler et al., 1985) and to act as an initiator of certain maturation processes of mouse mast cell clones (Galli et al., 1983).

Characteristic features of the transglutaminase reaction, as determined by measurement of the incorporation of histamine into succinylated casein, were observed with the PT18 cell extract (Table I). The activity was calcium-dependent and was inhibited by iodoacetamide under conditions known to favor this reaction (Folk and Cole, 1966). Enzymatic activity could be completely removed from the cell extract by immunoprecipitation with the monospecific anti-transglutaminase antibody.

### Table I

| Experimental conditions | Transglutaminase activity (pmol/min/mg protein) |
|-------------------------|--------------------------------------------|
| I CaCl\(_2\) concentration (mM) | 0 | 0.05 | 0.1 | 0.5 | 1.0 | 2.0 |
| | 0 | 12 | 19 | 39 | 58 | 76 |
| II Iodoacetamide inactivation | 0 |
| III Depletion of transglutaminase by antibody | 0 |

*The assay conditions were as given under “Experimental Procedures” with the exception that in experimental condition I the CaCl\(_2\) level was varied.*

*Extract was preincubated with 0.2 mM iodoacetamide at pH 6.8 in 0.1 M Tris-acetate buffer containing 2 mM CaCl\(_2\).*

*Extract was treated for 4 h at 4 °C with affinity purified anti-transglutaminase antibody (20 μg/ml) and goat anti-rabbit IgG (which, itself, did not inhibit transglutaminase activity). The immune complexes were removed by centrifugation before assay.*

**Increase in Transglutaminase Activity in Stimulated Mast Cells**—Elevated levels of transglutaminase activity were observed in cell lysates prepared from mast cells previously activated to release histamine by either immunologic or non-immunologic stimuli (Fig. 2). Unsensitized cells showed a 2- to 7-fold increase in enzyme activity upon stimulation with ionophore A23187 or ionophore Br-x537A (Fig. 2A). ConA, which is capable of inducing histamine release from PT18 mast cells (Barrett et al., 1984) did not significantly increase transglutaminase activity. Cells passively sensitized with anti-ovalbumin IgE and challenged with ovalbumin showed the highest increase in transglutaminase activity (Fig. 2B). The concentration of immunologic and non-immunologic secretagogues used were those reported to induce maximum histamine release (Barrett et al., 1984), and enzyme activity was assayed 10 min after stimulation, when the histamine release was complete (Barrett et al., 1984).

**Labeled γ-Glutamlyhistamine in Proteins from Mast Cells Metabolically Labeled with \(^{3}H\)Histidine**—The methodology employed here for identification of protein-bound γ-glutamlyhistamine is similar to that which was used to identify γ-glutamlypolyamine derivatives in proteins (Folk et al., 1980). Briefly, the cellular protein fraction is subjected to exhaustive proteolytic digestion, and the covalently bound amine, released as a stable γ-glutamyl derivative, is separated chromatographically. For this approach to be valid it is essential that γ-glutamlyhistamine be stable to conditions used for proteolytic digestion of the protein fraction, that the digestion conditions yield the expected derivative from the protein-amine conjugate, and that formation of γ-glutamlyhistamine does not occur as an artifact of the digestion procedure. As with the γ-glutamlypolyamines, synthetic γ-glutamlyhistamine was found stable to digestion, and essentially quantitative release of radiolabeled γ-glutamlyhistamine was observed upon digestion of a \(^{3}H\)histamine-casein conjugate prepared as outlined under “Experimental Procedures.” When the digestion protocol was carried out using unmodified casein or...
Transglutaminase and γ-Glutamylhistamine in Mouse Mast Cells

Fig. 2. Transglutaminase activity in cell lysates of mouse mast cells before and after histamine release. Transglutaminase levels are given as means ± S.E. of five determinations. The data for per cent of total cell histamine released are those obtained from separate experiments. A, unsensitized cells; B, IgE-sensitized cells. Ag is aggregated ovalbumin, the antigen in this system.

extracts from mast cells grown without radiolabel under the same conditions and to which labeled histamine was added at the beginning of the digestion, no γ-glutamylhistamine was found. Thus, the occurrence of γ-glutamylhistamine in protein digests is strong evidence for covalent conjugation of histamine through γ-glutamyl linkage in these proteins.

Our first approach in an effort to obtain evidence for covalent incorporation of histamine into cellular protein was to label histamine metabolically through growth of mouse mast cells in the presence of [3H]histidine. This, of course, also results in radiolabeling of most, if not all, of the cellular proteins with [3H]histidine. Because γ-glutamylhistamine is not separated from histidine in our ion exchange chromatographic system (Fig. 3A), direct identification of γ-glutamylhistamine by this procedure was impossible. As an indirect approach, a portion of the digest from labeled cells that had been stimulated immunologically was treated with the enzyme γ-glutamylamine cyclotransferase in order to release free histamine from the γ-glutamyl derivative. This enzyme has been shown to catalyze production of 5-oxoproline and free amines from γ-glutamylamines (Fink et al., 1980). A small increase in the amount of free histamine could be detected in this way (Fig. 3B). However, owing to the fact that there was always some unconjugated labeled histamine nonspecifically bound to the trichloroacetic acid-precipitated proteins and that this histamine was released during digestion, this approach provided only a suggestion of the presence of the γ-glutamyl derivative. Circumstantial evidence was obtained by collecting fractions corresponding to the γ-glutamylhistamine region of the chromatogram, treating them directly with γ-glutamylamine cyclotransferase, and rechromatographing (Fig. 3C). In this case counts eluted at the position of histamine were found to be in good agreement with the increased number of counts observed in the previous case, strongly suggesting the presence of γ-glutamylhistamine in the digest.

Positive identification was made by the use of a reverse phase high performance liquid chromatography system in which a separation of histidine and γ-glutamylhistamine was accomplished (Fig. 4). Fig. 5A shows the separation obtained with the digest of cell proteins prepared from metabolically labeled, immunologically stimulated mast cells. Here, a distinct peak of radioactivity is observed at the position of γ-glutamylhistamine. This material was collected and examined by chromatography in the ion exchange system described in Fig. 3. The radioactivity was found to elute exclusively at the position of synthetic γ-glutamylhistamine (Fig. 5B). Treatment with γ-glutamylamine cyclotransferase provided the final proof of its identity (Fig. 5C).

γ-Glutamylhistamine in Proteins from Mast Cells Activated in the Presence of [3H]Histamine—Because of the abundance of radiolabeled histidine in proteins of cells metabolically labeled with [3H]histidine, it was not possible to identify the protein or proteins that contain radioactivity in the form of histamine. In an attempt to overcome this problem [3H]histamine (2 μCi/ml) was supplied externally to cells immediately prior to stimulation. Ten minutes after the addition of secretagogues, a portion of the cells was precipitated by the addition of trichloroacetic acid and digested proteolytically as described. Another portion of these cells was collected by centrifugation, resuspended in TBS, denatured, and analyzed by SDS-gel electrophoresis. The digest prepared from immunologically stimulated cells contained a detectable amount of radioactivity that chromatographed at the position of γ-glutamylhistamine as shown in Fig. 6A. In addition to this component and histamine, small amounts of other radioactive materials were observed. These may result from oxidation of histamine by amine oxidases present in mast cells (Beaven, 1982). Examination of SDS-gels prepared from samples of undigested cell protein showed a radiolabeled component(s) that did not enter the stacking gel (Fig. 6B).

Level of γ-Glutamylhistamine in Mast Cells—Table II shows the content of γ-glutamylhistamine in cells before and after stimulation. Included are results obtained with cells metabolically labeled with [3H]histidine and those labeled by treatment with radiolabeled histamine. In each case data were obtained for both immunologic and nonimmunologic stimulation and in each case the greatest incorporation was observed after immunologic stimulation.

Association of Both Transglutaminase and γ-Glutamylhistamine with the Cellular Protein Fraction following Cell Stimulation—Table III summarizes the results of an experiment in which transglutaminase activity and the level of γ-glutamylhistamine were measured both in the cells and in the noncellular fraction following cell stimulation. Clearly, the majority of both enzyme activity and γ-glutamylhistamine remain associated with the cells. It was also observed that when unlysed cells were assayed for transglutaminase activity, a significant proportion of total enzyme activity was expressed, but only after cell stimulation.

γ-Glutamylhistamine was not detected in the undigested...
Trichloroacetic acid-soluble fraction of either the cellular pellet or the released material in these experiments, suggesting that there is no mechanism for formation of free γ-glutamylhistamine in these cells.

**DISCUSSION**

The main purpose of this study was to provide evidence for transglutaminase-catalyzed production of covalent protein-bound histamine conjugates in cells. Indeed, the results clearly show that, when PT18 mouse mast cells, in which the transglutaminase gene is expressed, are triggered to release histamine, there is an increase in transglutaminase activity accompanied by an increased formation of γ-glutamylhistamine in cellular protein (Fig. 2 and Table II). Although this is not direct proof that histamine incorporation into protein is a cellular function for transglutaminase(s), serious consideration must be given to this possibility.

The facts (i) that before stimulation, mast cells, which possess very high levels of histamine, are found to contain only small amounts of γ-glutamylhistamine and (ii) that upon stimulation, these cells lose a large percentage of their histamine and at this time display increases in both transglutaminase activity and γ-glutamylhistamine are consistent with a suggestion that stimulation to release histamine or its release per se provides a specific biochemical signal for catalytic incorporation of histamine into cellular protein. That this catalytic step is not simply the consequence of a signaled increase in transglutaminase activity seems evident because the mast cells contain significant amounts of this enzyme activity before stimulation (Fig. 1 and Table III).

The recent reports of a highly reactive intramolecular γ-glutamyl thiol ester in certain proteins, albeit extracellular ones, and of its reaction with primary amines to yield protein-bound γ-glutamylamines (Tack et al., 1980; Van Leuven, 1984) demand consideration of this type of noncatalytic reaction as an alternative mechanism for attachment of histamine to cellular protein. It is clear, therefore, that our findings of enhanced transglutaminase activity and production of protein-bound histamine do not provide the evidence sought to define an important intracellular role for transglutaminase(s). They are, however, unique in showing concomitancy of these two cellular events. There have been a number of reports of increases in cellular transglutaminase activity following stimulation of immunocompetent cells, e.g. human peripheral blood lymphocytes (Novogrodsky et al., 1978), antigen-specific B lymphocytes (Julian et al., 1983), and monocytes and macrophages (Fesus et al., 1981, Leu et al., 1982) and several papers present data on the occurrence of γ-glutamylamines in cellular proteins (Folk et al., 1980; Fan et al., 1983; Cariello et al., 1984). To our knowledge this is the first report in which the enzyme and its possible product have been shown to increase concurrently upon stimulation of cells. It is significant in this connection that in 1963 Ginsburg et al. reported a definite increase in both transglutaminase activity and protein-bound histamine in the livers of endotoxin-treated mice.

**FIG. 3.** Ion exchange chromatographic properties of histidine, γ-glutamylhistamine, and histamine. Chromatography was carried out according to a published procedure (Folk et al., 1980) except for omission of the first buffer of the five-buffer system. Fractions were collected for 1.5 min. A, distribution of radioactivity from samples of [3H]-labeled histidine (●) and of [3H]-labeled γ-glutamylhistamine, before (○) and after (□) its incubation with the enzyme γ-glutamylamine cyclotransferase. The arrow indicates the position of histamine. B, distribution of radioactivity in a digest of the protein fraction from mast cells, before (○) and after (□) its incubation with γ-glutamylamine cyclotransferase. C, distribution of radioactivity after incubation of fraction 17 from B (●) γ-glutamylamine cyclotransferase. The cells were metabolically labeled with [3H]histidine and release of histamine was triggered immunologically. In B, incubation with γ-glutamylamine cyclotransferase was carried out for 2 h at pH 7.0 in 0.3 M sodium phosphate buffer using the digest from approximately 1.2 mg of cell protein and 3.0 units of enzyme in 1 ml. In C, treatment with enzyme was under the same conditions of pH and enzyme. The pH of fraction 17 was adjusted to pH 7.0 before addition of buffer and enzyme.
mice that were administered high levels of histamine. Even at this early date the question was raised whether there is a causal connection between the increase in enzyme and the increased histamine incorporation (Waelsch, 1962). A quotation from Waelsch's paper is relevant to our present finding: "...does transglutaminase participate in the histamine fixation in vivo? A direct proof is difficult, and the isolation of the corresponding γ-glutamylhistamine would provide only further circumstantial evidence."

The level of protein-bound γ-glutamylhistamine found here in metabolically labeled mast cells after stimulation (350–500 pmol/mg protein, Table II) may be compared with that reported by Ginsburg et al. for histamine covalently attached to liver protein of endotoxin-treated mice after their injection with histamine (3–5 pmol/mg protein) assuming that this latter value was, indeed, a measure of histamine conjugated in γ-glutamyl linkage. The considerably higher level found in the stimulated mast cells is perhaps not surprising since these cells are rich in the amine. In vivo, histamine may be incorporated into protein only within cells and/or only in certain cells may incorporation occur. In this respect, fibroblasts (Chinese hamster ovary cells), which contain a high level of transglutaminase (Davies et al., 1984; Fesus et al., 1985), when cultured in a medium containing radiolabeled histamine, are found to internalize the amine, but to produce no detectable protein-bound γ-glutamylhistamine. It is perhaps significant in this regard that production of the covalently bound histamine derivative is measurable in these cells following an increase in transglutaminase activity (particularly in the cell membrane fraction; Fesus et al., 1985) induced by the tumor promoter phorbol ester.

After mast cell activation a significant portion of the transglutaminase activity can be detected without lysis of the cells (Table III). Because little enzyme activity is found in the noncellular fraction following activation, there is reason to believe that a significant amount of enzyme becomes oriented on the outer surface of these cells. There are reports of transglutaminase activity on the surface of human macrophages (Murtaugh et al., 1983b) and in association with microvesicles shed from the surface of tumor cells (Fesus et al., 1985). It is possible that in vivo mast cells supply both enzyme and amine substrate in the proper orientation for extracellular production of protein bound γ-glutamylhistamine.

Free γ-glutamylhistamine occurs as a major product of histamine metabolism in the ganglia of marine mollusks (Weinreich, 1978) and free γ-glutamylhistamine is found in rat brain after intraventricular injection of histamine (Kishi and Kakimoto, 1976). The mechanism of its formation is not understood. The absence of free γ-glutamylhistamine in mast cells either before or after stimulation is evidence that these cells provide no means for conjugation of histamine in this manner.

Among the several theories regarding the physiological role

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Fig. 5. Identification of γ-glutamylhistamine in the digest of a protein fraction from PT18 cells that had been stimulated immunologically. Prior to high performance liquid chromatography, a 2.0-ml portion of the digest (approximately 5.2 mg of protein) was applied to a 0.8 × 5-cm column of Bio-Rex 70 resin (NH₄⁺ form adjusted to pH 4.7 with acetic acid). Neutral and acidic components were washed from the resin with water, and γ-glutamylhistamine was eluted with 1.0 N NH₄OH. The eluate was evaporated under a stream of nitrogen and dissolved in water for analysis. A, high performance liquid chromatographic distribution of radioactivity from the digest. The chromatographic conditions were those described in Fig. 4. B, ion exchange chromatography of the γ-glutamylhistamine fraction separated by high pressure liquid chromatography in A. The conditions for chromatography were as outlined in Fig. 3. C, ion exchange chromatography of the γ-glutamylhistamine fraction separated as in A and B after treatment with the enzyme γ-glutamylamine cyclotransferase (conditions as described in Fig. 3).

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8 L. Fesus, unpublished observation.
The quantities of γ-glutamylhistamine were calculated based on the specific radioactivity of intracellular histamine. Specific radioactivity in the trichloroacetic acid supernatant from washed cells was determined from fluorometric measurements of histamine (Shore et al., 1959) and measurements of histamine radioactivity following separation by ion exchange chromatography. Full recovery of covalently bound histamine as γ-glutamylhistamine was indicated by the facts that essentially all of the radioactivity in digests was accounted for in the protein fraction of mast cells triggered to release histamine in the presence of [3H]histamine. The arrows designate the positions of γ-glutamylhistamine and histamine, respectively. The upper chromatogram is that obtained upon treatment of the γ-glutamylhistamine fraction of the first chromatogram with the enzyme γ-glutamylmamyl cyclotransferase (condition of Fig. 3). B, SDS-gel electrophoresis and autoradiography were performed as described in Fig. 1. In lane 1 are shown the positions of protein standards as determined in Fig. 1 (molecular weights are given in kilodaltons; lane 2, 200 μg of protein from unstimulated cells that were incubated with [3H]histamine for 10 min before preparation for electrophoresis; lane 3, 100 μg of protein from cells stimulated immunologically; lane 4, 100 μg of protein from cells stimulated with the calcium ionophore A-23187.

**Table II**

| Stimuli         | In protein of cells labeled metabolically with [3H]histidine (pmol/mg protein) | In protein of cells incubated with [3H]histamine (pmol/mg protein) |
|-----------------|---------------------------------------------------------------------------------|------------------------------------------------------------------|
| None            | 16                                                                              | Undetectable                                                   |
| Antigen         | 589                                                                             | 121                                                             |
| Ionophore A23187| 334                                                                             | 34                                                              |

The amounts of γ-glutamylhistamine were determined as outlined in Table II using cells metabolically labeled with [3H]histidine.

**Table III**

| Cell stimulation | Transglutaminase activity* | γ-Glutamylhistamine* (%) |
|------------------|----------------------------|--------------------------|
| Nonstimulated cells | 87 (2.0)                | 92                       |
| Immunologic stimuli | 91 (27)                   | 86                       |
| Ionophore A23187 | 83 (23)                   | 94                       |

*Cells were incubated with or without stimuli under the conditions outlined under “Experimental Procedures.” After 10 min, the cells and the noncellular fraction were separated by centrifugation (5 min at 2900 × g).

Transglutaminase activity was measured in an experiment in which nonradiolabeled cells were used. Measurements were made before and after lysis of cells. The values given in parentheses are those for the per cent of the total enzymatic activity found with unlysed cells.

The amounts of γ-glutamylhistamine were determined as outlined in Table III using cells metabolically labeled with [3H]histidine.

The possible differential in incorporation of histamine and entry of labeled amine, the present finding of a histamine-containing high molecular weight protein may reflect only the latter stage of the overall reaction. It is quite possible that this high molecular weight protein is produced by transglutaminase action, if indeed enzyme-catalyzed cross-linking through ε-(γ-glutamyl)lysine bonds occurs during histamine release. In any case, both transglutaminase and the protein(s) that contain the γ-glutamylhistamine remain within the cells suggesting that their roles, whatever they may be, are essential.

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