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Evidence for participation of transglutaminase in receptor-mediated endocytosis

(receptor/clustering/glutaminyl-peptide γ-glutamyltransferase)

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ABSTRACT **We report evidence that the enzyme transglutaminase (glutaminyl-peptide γ-glutamyltransferase; R-glutaminyl-peptide:amine γ-glutamyltransferase, EC 2.3.2.13) participates in receptor-mediated endocytosis. Clustering and internalization of rhodamine-labeled α_2-macroglobulin (Rα_2M) in normal rat kidney (NRK) cells is inhibited by a wide spectrum of compounds that inhibit transglutaminases, including that from NRK cells. The pattern of clustering inhibition resembles the pattern of transglutaminase inhibition as follows: (*i*) The most potent transglutaminase inhibitors are dansylcadaverine and the transglutaminase-directed affinity label *N*-benzyloxycarbonyl-5-diazo-4-oxonorvaline *p*-nitrophenyl ester; these were also the most potent inhibitors of clustering and internalization of Rα_2M. (*ii*) The inhibition of clustering of Rα_2M occurs in the same concentration range as that required for transglutaminase inhibition. (*iii*) Linear primary amines are more effective blockers than the iso-chain primary amines. (*iv*) The transglutaminase affinity label *N*-benzyloxycarbonyl-5-diazo-4-oxonorvaline *p*-nitrophenyl ester irreversibly inhibits a significant fraction of the NRK transglutaminase and the clustering and internalization of Rα_2M. A closely related compound, *N*-trifluoroacetyl-6-diazo-5-oxonorleucine ethyl ester, does not significantly inhibit transglutaminase or clustering and internalization. (*v*) Clustering and internalization is inhibited 10-fold more effectively by the heptapeptide Ac-Gly$_2$-LLeu-LLys-Gly$_3$ than by the heptapeptides Ac-Gly$_2$-LLeu-DLys-Gly$_3$ or Ac-Gly$_3$-DLys-DLeu-Gly$_2$. This is the pattern of stereospecificity for the inhibition of purified transglutaminases.**

The binding of certain ligands, such as polypeptide hormones, to specific cell surface receptors is followed in many instances by the aggregation of the ligand–receptor complexes into a cluster (1–3). The clustering event occurs within the domain of "coated pits," which may then pinch off to form endocytic vesicles. This type of concentrative adsorptive endocytosis seems to be a general mechanism for the internalization of certain hormones (1–3), low density lipoproteins (4), lysosomal hydrolases (5), and probably many other proteins. By using fluorescent derivatives of insulin and α_2-macroglobulin (α_2M), the time course of the formation of these clusters and their internalization have been followed (1–3, 6). Recently we observed (7, 8) that amines (7) and certain lysine- and glutamine-containing peptides (8) block clustering without inhibiting the ligand–receptor binding process itself. Removal of the inhibitory amine allows the bound ligand to cluster and be internalized, indicating that the inhibitory action of the amine is reversible. These findings have suggested to us that the formation of clusters of receptor–ligand complexes in coated pits is mediated by the enzyme transglutaminase (TGase; glutaminyl-peptide γ-glutamyltransferase; R-glutaminyl-peptide:amine γ-glutamyltransferase, EC 2.3.2.13) (7). TGases catalyze the formation of ε-(γ-glutamyl)-lysine between protein molecules,

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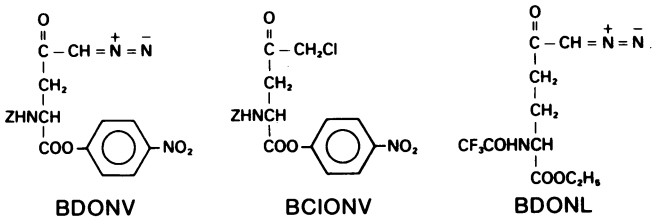

FIG. 1. Structures of BDONV, BClONV, and BDONL. Z, benzyloxycarbonyl.

and also catalyze the coupling of amines and diamines to the γ-carboxyl residue of glutamine. All known transglutaminases are Ca^{2+} dependent and are inhibited by aliphatic amines (9). In this report, we explore in more detail the possible involvement of TGase in the clustering of receptor–ligand complexes. We examined a wide spectrum of TGase inhibitors for their ability to inhibit the clustering and internalization of rhodamine-labeled α_2M (Rα_2M) on normal rat kidney (NRK) cells, and we examined in parallel the potency of these compounds as inhibitors of the TGase activity of NRK cells.

MATERIALS AND METHODS

Materials. Blocked diazooxonorvaline (BDONV), blocked chlorooxonorvaline (BClONV), and numerous other TGase inhibitors were obtained through the generosity of J. F. Folk (National Institutes of Health). BDONL was obtained from Cordova Chemical Company (CA) (see Fig. 1). Dansylcadaverine (puriss, p.a.) was obtained from Fluka (Buchs, Switzerland). Rα_2M was prepared as described (10). [^{3}H]Putrescine was obtained from New England Nuclear.

Growth of Cells. NRK cells were grown in 35-mm dishes or roller bottles in Dulbecco–Vogt's medium with 10% heated calf serum. For fluorescence microscopy, cells were planted on 35-mm (9.6-cm^2) dishes at a density of 5×10^3 to 1×10^4 cells per dish and were used 3–4 days later at 60–80% confluency. Under these conditions the cells are firmly attached to the dish and are not removed or lysed by treatment with 8–10% (vol/vol) dimethyl sulfoxide (Me_2SO), the concentration required to render the cells permeable (11, 12) to the various compounds tested (see below). To examine the TGase activity, cells were grown in roller bottles (850 cm^2). Cells were planted at 1×10^7 cells per bottle, the medium was changed every 2 days, and the cultures became confluent after 5–6 days.

Abbreviations: α_2M, α_2-macroglobulin; Rα_2M, rhodamine-labeled α_2M; Me_2SO, dimethyl sulfoxide; BDONV, blocked diazooxonorvaline (*N*-benzyloxycarbonyl-5-diazo-4-oxonorvaline *p*-nitrophenyl ester); BClONV, blocked chlorooxonorvaline (*N*-benzyloxycarbonyl-5-chloro-4-oxonorvaline *p*-nitrophenyl ester); BDONL, blocked diazooxonorleucine (*N*-trifluoroacetyl-6-diazo-5-oxonorleucine ethyl ester); TGase, transglutaminase; P_i/NaCl, 0.01 M sodium phosphate, pH 7.4/0.15 M NaCl; Z, benzyloxycarbonyl.

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Inhibition of Clustering and Fluorescence Microscopy. Medium was removed from 35-mm dishes and the cells were rinsed twice with serum-free medium (Dulbecco–Vogt) at 37°C. Then each dish was incubated with serum-free medium with or without 8–10% Me_2SO and the desired inhibitor at 37°C for 30 min. The Me_2SO-containing medium was employed when the cells were impermeable to the compound tested. Controls in the presence of serum-free medium and Me_2SO-containing medium were performed in parallel. The Me_2SO-containing medium was prepared immediately prior to the experiment. After 30 min, the medium containing the inhibitor was removed and the cells were washed twice with serum-free medium at 37°C. Subsequently, the cells were incubated with Rα_2M at 40 μg/ml (51 nM) and inhibitor in serum-free medium for 20 min at 37°C, always without Me_2SO. Then the Rα_2M-containing medium was removed, and the cells were washed three times with 1.5 ml of serum-free medium (37°C) and once with phosphate-buffered saline (P_i/NaCl) (37°C), then fixed with 2% (wt/vol) formaldehyde in P_i/NaCl for 1 min at room temperature and washed with P_i/NaCl. The cells were mounted under a circular coverslip in glycerol medium and examined by using a Zeiss (RA) microscope equipped with rhodamine epifluorescence optics and a silicon intensifier target television camera. Pictures were usually taken directly from the television screen with a Polaroid camera, using the same settings for all pictures.

Treatment of NRK Cells with BDONV, BClONV, or BDONL. The compounds were dissolved in Me_2SO such that upon addition to the cells the Me_2SO concentration would not exceed 1%. The cells were incubated with the desired concentration of either of the compounds for 30 min at 37°C. Then the medium was removed by suction and the cells were washed twice with Dulbecco–Vogt serum-free medium at 37°C (2 ml per 35-mm dish and 100 ml per roller bottle). The cells were incubated with Rα_2M for 20 min at 37°C as usual and processed for fluorescence microscopy as described above. Extracts were prepared from the NRK cells grown in roller bottles for measurement of TGase activity as described below.

NRK Extracts. NRK cells were grown in roller bottles to 80–100% confluency. The medium was removed and the cells were washed twice with 100 ml of cold 20 mM Tris·HCl, pH 7.4, containing 0.15 M NaCl or with 100 ml of P_i/NaCl. Then 40 ml of P_i/NaCl containing 1 mM EDTA was added, and each bottle was incubated for 3–5 min at 37°C until all the cells had detached. The cells were centrifuged at 700 × *g* for 5 min and resuspended in 3–4 ml per bottle of 20 mM Tris·HCl, pH 8.0 containing 1 mM EDTA and 1 mM dithiothreitol or 20 mM 2-mercaptoethanol. After incubation for 30 min to 2 hr at 4°C the cells were disrupted with a Teflon homogenizer (25–35 strokes). The resulting extract was used for the TGase assay. TGase activity was stable for many weeks when stored at −70°C or in liquid nitrogen. TGase activity was assayed by measuring [^{3}H]putrescine incorporation into casein (10). The assay mixture contained: casein (Sigma) at 2.0 mg/ml, 7.5 mM $CaCl_2$, 50 mM Hepes (pH 7.4), and 1.2 mM [^{3}H]putrescine (100–120 dpm/pmol).

RESULTS

TGase catalyzes the formation of an isopeptide bond between a glutaminyl side chain and an ϵ-amino side chain of proteins with the expulsion of 1 mol of ammonia per mol of isopeptide bond formed (9). TGase also catalyzes the reaction between glutamine residues in proteins and certain alkyl amines and arylalkylamines such as methylamine and dansylcadaverine, or diamines such as putrescine. The enzyme also uses short glutamine-containing peptides as substrates. We therefore examined the effect of several classes of TGase substrates on the clustering and internalization of Rα_2M: (*i*) amines, which bind to the enzyme's amine subsite; (*ii*) lysine-containing compounds, which also bind to the amine subsite; (*iii*) glutamine-containing compounds, which bind to the glutamine subsite; and (*iv*) two TGase affinity labels known to inhibit TGases irreversibly. NRK cells are impermeable to some of the compounds. Therefore, we had to find conditions that allowed the compounds to enter cells without disturbing the process of clustering and internalization.

Permeation of Cells. Me_2SO allows the insertion of impermeant small molecules into cells (11, 12). We treated cells with 8–10% Me_2SO and found that it allowed the impermeant dye trypan blue to enter the cells yet did not affect the ability of the cells to cluster and internalize Rα_2M. In the presence of Me_2SO, however, we were able to inhibit the clustering of Rα_2M and with a large variety of transglutaminase inhibitors that were ineffective in the absence of Me_2SO (see below). Compounds such as monoamines that possess a hydrophobic side chain and only one electrostatic charge penetrate cells in the absence of Me_2SO; with such compounds Me_2SO was omitted.

Inhibition of Rα_2M Clustering and Internalization by TGase Inhibitors. The inhibition of Rα_2M clustering was determined by counting the number of fluorescent spots per cell at various concentrations of the TGase inhibitors. The results are expressed as the concentration required to cause a 50% reduction in the number of fluorescent spots after 20 min. The spots that one observes after 20 min such as those shown in Fig. 2 *A* and *C* represent clusters of fluorescent ligands in small vesicles in the cytoplasm (2). The ligand enters these vesicles after initially clustering in coated pits. Many monoamines were found to inhibit Rα_2M clustering and internalization. The straight chain amines *n*-propylamine and *n*-pentylamine are more potent than the corresponding branched chain amines (Table 1). The most potent monoamine inhibitor is dansylcadaverine (Fig. 2); it is also the most potent TGase inhibitor (see below and Table 1).

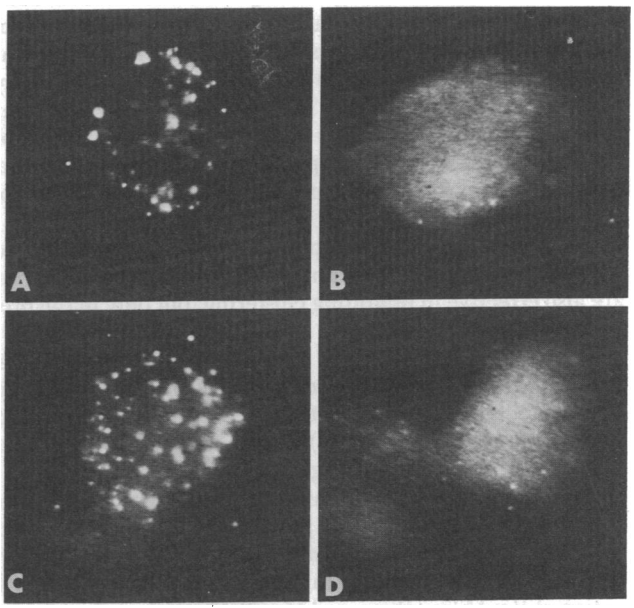

FIG. 2. Inhibition of Rα_2M clustering by BDONV and dansylcadaverine. NRK cells were treated with BDONV or BDONL for 30 min and washed extensively before Rα_2M was applied. When dansylcadaverine was used, the latter was added to the cells 10–15 min prior to the addition of Rα_2M. (*A*) Control; (*B*) 0.15 mM BDONV; (*C*) 2.5 mM BDONL; (*D*) 50 μM dansylcadaverine.

Table 1. Apparent dissociation constants of TGase substrate amines from TGase and their potencies in inhibiting Rα_2M clustering

Compound	$S_{0.5}$ for clustering inhibition,* mM	Apparent dissociation constant, mM, for TGase of NRK†	Guinea pig liver†	Human plasma Factor XIIIa‡
Simple amines				
Methylamine	12 ± 3.0	ND	15 ± 2	—
Ethylamine	1 ± 1	ND	1.6 ± 0.14	3.7 ± 0.10
1-Methylethylamine (isopropylamine)	10.0	ND	107 ± 10	115 ± 3.0
n-Propylamine	3 ± 1	ND	1.9 ± 0.06	2.9 ± 0.44
2-Ethyl-*n*-propylamine (isobutylamine)	5 ± 1	138 ± 40	67 ± 9.0	63 ± 7.0
n-Butylamine	0.75 ± 0.3	13.5 ± 2.7	5.2 ± 0.20	3.6 ± 0.09
3-Methyl-*n*-pentylamine	3.5 ± 1.5	ND	18 ± 1.30	5.0 ± 0.08
n-Pentylamine	0.75 ± 0.3	ND	4.7 ± 0.18	2.5 ± 0.08
n-Hexylamine	0.75 ± 0.3	ND	1.07 ± 0.12	231 ± 0.02
Aryl derivatized amines				
N-(ε-Aminocaproyl)-β-phenylethylamine	2.5 ± 0.5	10 ± 3.0	0.9 ± 0.09	ND
Dansylcadaverine	0.015 ± 0.05 (Rα_2M)	0.185 ± 0.04	0.0057§	ND
Other amines¶				
Putrescine	25 ± 5	13 ± 4	—	—
Histamine	1.5 ± 0.5	ND	—	—
Aminoacetonitrile	1.5 ± 0.5	ND	—	—
Glycinamide	1.5 ± 0.5	ND	—	—
Cystamine	3.0 ± 1.0	ND	—	—

ND, not determined. Values are mean ± SEM.

* $S_{0.5}$ is the concentration of ligand required to inhibit the formation of 50% of the patches upon incubation of NRK cells.

† Measured by inhibition of [^{3}H]putrescine incorporation into casein. Some of the data are depicted in Figs. 4 and 5.

‡ Determined from the inhibition of [^{14}C]methylamine incorporation into the acylated B chain of insulin at pH 7, 30°C. Data of Gross *et al.* (13). The fact that dansylcadaverine is a potent inhibitor of Factor XIIIa was already noted by Nilsson *et al.* (14) and analyzed in detail recently (15).

§ Determined from the inhibition of [^{14}C]methylamine incorporation into the acylated B chain of insulin at pH 7, 30°C. Data of J. E. Folk (personal communication).

¶ Inhibition by these compounds occurs only in the presence of 8–10% Me_2SO, which makes the cells permeable to these reagents. It is established that all these compounds serve as potent substrates for TGase (16–18), although the exact apparent affinities of these compounds to other TGases were not determined. For example, at 16 mM these compounds were found (18) to inhibit human lymphocyte TGase activity 90–98%.

Inhibition of Clustering in Me_2SO-Permeabilized Cells. Putrescine, histamine, aminoacetonitrile, and glycinamide are good substrates and inhibitors for TGase (9) and inhibit clustering in permeabilized cells but not in untreated cells. Lysine-containing peptides also inhibit the clustering and internalization, and they do so only in the presence of Me_2SO (Table 2). The inhibition is stereospecific; the L-lysine-containing peptide is at least 10 times more potent than the D-lysine-containing peptide.

Glutamine-containing peptides are good substrates for TGases and bind to the glutamine subsite of the enzymes. They are also effective in inhibiting clustering, but only in the presence of Me_2SO (Table 3).

Irreversible Inhibition of Clustering by TGase-Directed Affinity Label. Folk and colleagues have shown that the blocked chloroketone derivative of norvaline, methyl-*N*-(2-hydroxy-5-nitrophenylacetyl)-2-amino-4-oxo-5-chloropentanoate, irreversibly inhibits guinea pig liver TGase (20). A similiar compound and its diazoketone analogue (Fig. 1) were found to irreversibly inhibit purified TGase as well as NRK TGase (Fig. 3). The two potent TGase affinity labels BDONV and BClONV irreversibly inhibit the clustering and internalization of Rα_2M (see above and Fig. 2). BDONL under identical conditions inhibits NRK TGase only slightly (Fig. 3); BDONL does not inhibit the clustering and internalization of Rα_2M (Fig. 2).

Inhibition of NRK TGase Inhibitors. Previous studies have shown that the amines and glutamine derivatives examined in this study are substrates of TGases from various sources (see review ref. 2). In particular, the guinea pig liver enzyme and factor XIIIa were extensively studied, and their substrate specificities were analyzed in detail (for references see Tables 1–3). Because our studies were on NRK cells, whose TGase has not been studied, we analyzed the TGase activity of NRK ex-

Table 2. Apparent dissociation constants of lysine derivatives from TGase and their potencies in inhibiting Rα_2M clustering

Compound	$S_{0.5}$ for clustering inhibition,* mM	Apparent dissociation constant, mM, for TGase of† Guinea pig liver	Human plasma Factor XIIIa
Ac-LLys-OMe	1.0 ± 0.5	0.35 ± 0.02	0.65 ± 0.04
Tosyl-LLys-NH_2	2.0 ± 1.0	ND	ND
Ac-Gly_2-LLeu-LLys-Gly_3	0.6 ± 0.2	0.08 ± 0.004	0.13 ± 0.02
Ac-Gly_2-LLeu-DLys-Gly_3	8.0	8.97 ± 0.84	9.04 ± 1.24
Ac-Gly_3-DLys-DLeu-Gly_2	8.0	5.22 ± 0.4	8.57 ± 1.27

ND, not determined. Values are mean ± SEM.

* In the presence of 8% Me_2SO.

† Due to the rapid hydrolysis of these compounds by NRK extracts, the dissociation constants of NRK TGase could not be determined. Data given are from ref. 19.

Table 3. Inhibition of Rα_2M clustering by glutamine substrates and a glutamine affinity label

Compound	$S_{0.5}$ for clustering inhibition, mM
Z-Gln-Gly	1.5 ± 0.5*
HNPA-Gln-Gly†	6.0 ± 1.0*
BDONV‡	0.10 ± 0.03‡ (irreversible)

The effect of Z-Gln-Gly and of HNPA-Gln-Gly on the patching of Rα_2M was examined in the presence of Me_2SO, because the cells are otherwise impermeable to these compounds. BDONV was applied to the cells for 30 min at 37°C and then the excess was removed by extensive washing. Further details are given in the text. Values are mean ± SEM.

* The inhibition of clustering was examined in the presence of 8–10% Me_2SO to permeabilize the cells. The K_m for Z-Gln-Gly for guinea pig liver TGase is 8.3 ± 2.0 mM (ref. 18).

† Obtained from J. E. Folk. HNPA, 2-hydroxy-5-nitrophenylacetyl.

‡ See also Fig. 1. Treatment with BDONV (Fig. 3) or BClONV (data not shown) causes an irreversible inhibition of a significant fraction of the NRK TGase activity.

tracts and compared the potencies of the TGase inhibitors in inhibiting the NRK activity with their ability to inhibit known TGases, as well as with their potencies in inhibiting clustering of ligand–receptor complexes.

The order of potency of monoamines in the inhibition of NRK TGase as well as in the inhibition of Rα_2M clustering was found to be dansylcadaverine > *n*-butylamine > isobutylamine (Table 1 and Figs. 4 and 5). This pattern of inhibition was observed previously for pure TGases.

TGases possess a sulfhydryl group at the active site, and most likely an acylenzyme intermediate is formed upon interaction with the glutamine-containing substrate (9). BDONV and BClONV are effective affinity labels of the enzyme and irreversibly inhibit its activity (Fig. 3). When intact cells were treated for 30 min with BDONV, a significant fraction of the NRK TGase activity was irreversibly inhibited (Fig. 3) concomitant with the inhibition of clustering of Rα_2M (Fig. 2). Similarly, BClONV irreversibly inhibited the TGase activity of NRK cells, and the clustering of Rα_2M (not shown). Interestingly, the diazoketone BDONL does not inhibit the NRK TGase activity under these conditions (Fig. 3) and does not

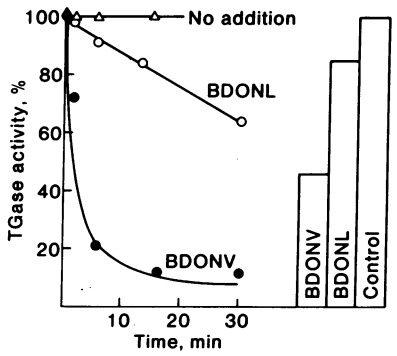

FIG. 3. Irreversible inhibition of NRK and guinea pig liver TGases by BDONV and BDONL. On the left is shown the effect of these diazoketones on purified guinea pig liver TGase. On the right is shown the effect of 0.2 mM BDONV and BDONL on NRK TGase activity after a 20-min treatment of whole cells followed by extensive washing. When the NRK cells were exposed to 0.5 mM BDONV the inhibition of TGase was 75%. Exposure of NRK cells to BDONL at concentrations up to 2.5 mM did not increase the inhibitory effect over that of 0.2 mM.

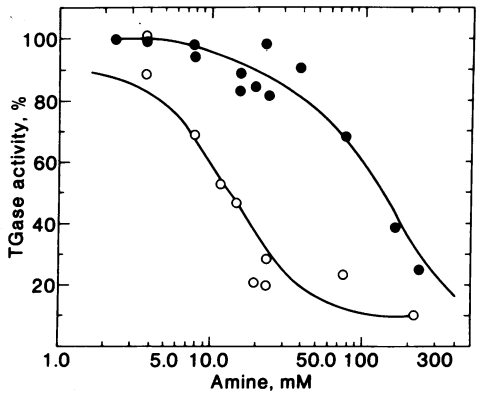

FIG. 4. Inhibition of NRK TGase by *n*-butylamine (○) and isobutylamine (2-methyl-*n*-propylamine) (●). TGase assays were performed with 1.1 mM [^{3}H]putrescine and increasing concentrations of the amines.

inhibit clustering of Rα_2M (Fig. 2). BDONL is a weaker inhibitor of guinea pig TGase than BDONV is (Fig. 3).

DISCUSSION

In this study we have explored in detail the hypothesis that TGase is involved in the clustering and internalization of ligand–receptor complexes. The approach taken was to study a large number of TGase inhibitors as potential inhibitors of clustering and internalization of Rα_2M in NRK cells. In parallel, many of the compounds were tested as inhibitors of the TGase activity of NRK cells. A large variety of TGase inhibitors that differ in their affinities for well-characterized TGases were found to inhibit the clustering and internalization of Rα_2M (Tables 1–3). The relative effectiveness of these compounds in inhibiting clustering was qualitatively similar to their affinity for known TGases and for the NRK TGase. The prominent features of the pattern of inhibition can be summarized as follows: (*i*) The inhibition of ligand–receptor clustering is stereospecific. For example, the stereoisomers that exhibit higher affinity for TGases also exhibit higher potency in the inhibition of clustering, and the heptapeptide Ac-Gly_2-LLeu-LLys-Gly_3 is much more potent than its L, D and D, D analogues (Table 2). (*ii*) Straight-chain monoamines are more potent than their branched-chain isomers (Table 1). (*iii*) Dansylcadaverine is the most potent TGase inhibitor of the monoamine class (Table 1) and is also the most effective inhibitor of Rα_2M clustering and internalization (Fig. 2, Table 1). (*iv*) BDONV and BClONV, which are site-specific TGase affinity labels, also block irreversibly the clustering and internalization of Rα_2M (Fig. 2,

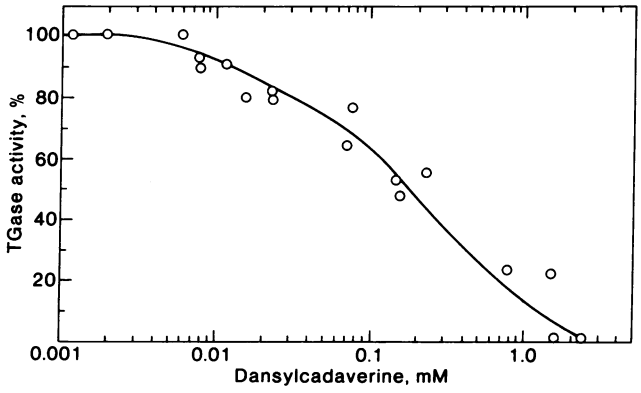

FIG. 5. Inhibition of NRK TGase by dansylcadaverine. Experimental conditions were as described for Fig. 4.

Table 3). Another diazoketone, BDONL, which is a fair inhibitor of guinea pig liver TGase (Fig. 3), is a poor inhibitor of TGase activity of NRK cells and has no effect on the clustering and internalization of Rα_2M under our experimental conditions.

Clustering Inhibition by Impermeant Compounds. The ability of certain TGase inhibitors to block the clustering of Rα_2M could be examined only after the cells had been made permeable by Me_2SO. In permeabilized cells, compounds such as putrescine, benzyloxycarbonylglutaminylglycine, *N*-(2-hydroxy-5-nitrobenzoyl)-glutaminylglycine, lysine-containing heptapeptides, *N*-acetyllysine methyl ester, tosyllysine amide, histamine, glycinamide, and aminoacetonitrile inhibit Rα_2M clustering.

Possible Involvement of Sulfhydryl Groups. Cystamine, a disulfide-containing compound, inhibits Rα_2M clustering and internalization at 5 mM. Cystamine is a substrate for TGase and can potentially interact with its active-site sulfhydryl group and induce inactivation. Nevertheless, this effect on clustering could be indirect.

Quantitative Differences. A close look at Table 1 reveals that there are some quantitative discrepencies between the apparent affinities of certain compounds for known TGases and their potencies in inhibition of clustering. For example, the straight-chain alkyl amines are equipotent in inhibiting TGase, whereas their potency in inhibiting clustering increases sharply with chain length. Also, the branched-chain amines are more effective in inhibiting clustering than in inhibiting TGases. The origin of these quantitative discrepencies is not yet known to us. The comparison made is between a complex cellular event and a well-defined enzyme reaction. The concentration of the amine in the medium may be very different from that inside the cell, where it acts. More studies are needed to establish the nature of these quantitative discrepancies. Another important point is that the TGase activity is assayed with casein as the glutamine-containing substrate, in the presence of numerous NRK cellular proteins and other potential TGase substrates such as putrescine. All these proteins possess lysine side chains that can serve as the amine substrate for the TGase. Furthermore, casein itself contains lysine residues.

Thus the externally added amine does not challenge the naked NRK TGase but rather competes with endogenous substrates. The dissociation constants of NRK TGase and the amines most likely represent "apparent" dissociation constants, and some of the discrepancies between the dissociation constants for the NRK TGase and purified TGase may be only "apparent." The concentration dependence of the amine-induced inhibition of clustering may reflect the effectiveness of the added amine in competing with endogenous amines such as lysine side chains and putrescine for the intracellular TGase.

Conclusion. Presently, we can summarize our hypothesis on the role of TGase in the process of receptor–ligand clustering as follows: The binding of ligand to a surface receptor causes a structural change in the receptor such that the receptor–ligand complex becomes trapped in a coated pit. This step is irreversible, and is blocked by TGase inhibitors. We propose that this step involves covalent bond formation catalyzed by the enzyme TGase. This covalent bond can be either a γ-glutamyl-ϵ-aminolysine isopeptide bond between two polypeptide sequences or γ-glutamylputrescine. The formation of a covalent bond probably commits the internalization reaction to proceed. At present, no direct evidence for such a covalent bond is available, nor have the endogenous molecules that serve as substrates for the TGase reaction been identified.

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