Human β_2-microglobulin is a Substrate of Tissue Transglutaminase: Polymerization in Solution and on the Cell Surface

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ABSTRACT Incubation of purified human β_2-microglobulin (B_2-m) with tissue transglutaminase (Tgase) resulted in the formation of high molecular weight polymers revealed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In the presence of 30 mM [^{14}C]methylamine, the polymer formation was prevented, but incorporation of methylamine into β_2-m (equal to 1 methylamine per 1 molecule) could be observed. From the sheddings of peripheral blood mononuclear cells occurring in the presence of Tgase, it is apparent that anti-β_2-m immunoadsorbent removed, in addition to human leukocyte antigen (HLA) and β_2-m, some other proteins. The enzyme could incorporate [^{14}C]methylamine into β_2-m of the shedding cells. On addition of rabbit anti-human β_2-m antibody, followed by fluoresceine-labeled goat anti-rabbit IgG antibody to human mononuclear blood cells, the otherwise homogeneous distribution of fluorescence turned into spots and patches on cells previously incubated with Tgase or Ca^{2+}-ionophore A23187.

Transglutaminases (Tgase) catalyze a calcium-dependent acyl-transfer reaction between the γ-carboxyamide group (acyl donors) of a peptide-bound glutamine residue and various primary amines (9). When peptide-bound lysine residues serve as acyl acceptors, inter- or intramolecular ε-(γ-glutamyl) lysine crosslinks are formed. Tgase activity as well as the crosslink is found to be widespread in cells and body fluids (10). However, only two physiological events in the mammalian system have been shown to result from a Tgase-catalyzed reaction (10): crosslinking of fibrin and the clotting of rodent seminal plasma (both occur extracellularly). A role for Tgase has been postulated in receptor-mediated endocytosis (7) and in regulating tissue proliferation through polyamines (10). However, the evidence is only circumstantial. Except in the case of fibronectin (1), the isolation of substrates and the demonstration of enzyme-catalyzed modifications have not been achieved.

β_2-microglobulin (β_2-m), a low molecular weight protein found on the cell surface of mammalian cells, is characterized by its striking similarity to the constant domains of immunoglobulins and its association with the major histocompatibility antigen (6, 11). Its primary amino acid sequence is known, and, out of 100 amino acids, it contains twelve glutamine and eight lysine residues. For this reason, we suspected that β_2-m might be a substrate of Tgase and we examined this question by three experimental approaches.

MATERIALS AND METHODS

Human β_2-microglobulin (β_2-m) isolated from culture media of human lymphoid cell lines was a kind gift of Dr. N. Tanigaki (Roswell Park Memorial Institute, Buffalo, New York). Guinea pig liver Tgase, which was purified according to Connellan et al. (5), exhibited 92% ± 8% of the reported specific activity upon assay by hydroxamate formation with benzyloxycarbonyl (2)-L-glutaminylglycine. Active site-inhibited Tgase prepared as previously described (15) showed no activity. An IgG fraction of monospecific rabbit anti-human β_2-m-antiserum was obtained from Dako Immunoglobulins Ltd. (Copenhagen, Denmark) and diluted to 700 μg/ml with Parker medium TC-199 just before use. Fluoresceine-labeled IgG of goat antiserum against rabbit IgG (Hyland Diagnostics Div., Travenol Laboratories, Inc., Costa Mesa, Calif.) was used as a second antibody at a concentration of 500 μg/ml. Ca^{2+}-ionophore A23187, a gift of Eli Lilly and Company (Indianapolis, Indiana), was dissolved in dimethylsulfoxide at 1 mg/ml and stored at 4°C. [^{14}C]Methylamine (specific activity 40 mCi/mmol) was purchased from New England Nuclear (Boston, Massachusetts). Protein standards for molecular weight determination by sodium dodecyl sulfate (SDS) gel electrophoresis were obtained from Serva Fine Biochemicals, Inc., Garden City Park, N.Y. All other chemicals were either reagent grade or the best available.

Crosslinking of β_2-m by Tgase

At various intervals after the addition of 3μl of Tgase (11 mg/ml) to a 150-μl solution of β_2-m (1.26 mg/ml in 30 mM Tris, pH 7.5, 100 nM NaCl, 5 mM $CaCl_2$, 2 mM DTT, 1 EDTA), 20-μl aliquots were withdrawn to process for SDS polyacrylamide get electrophoresis (PAGE) on disc gels (17). When the incorporation of labeled methylamine into β_2-m was studied, 30 mM [^{14}C]methylamine was also included in the incubation mixture. After the usual electrophoresis, staining, and destaining procedures, the band corresponding to β_2-m was cut out

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from the gel, sliced, solubilized in Soluene-350 (Packard Instrument Co., Inc., Downers Grove, Ill.), and radioactivity was measured in a Packard liquid scintillation counter type 3320.

Preparation of Human Peripheral Blood Mononuclear Cells

Human peripheral blood mononuclear cells (PBMC) were separated from freshly drawn blood of healthy adults with a Ficoll-Uromiro (Pharmacia Uppsala, Sweden) gradient according to the method of Böyum (2). The preparation contained 90–95% lymphocytes as determined by morphology, <3% of the cells engulfed latex particles, and 98% of the cells were viable as indicated by trypan blue exclusion.

Shedding of Surface-labeled Human PBMC and Adsorption of the Shed Supernate on Sephadex Anti-β_2-m Particles

Supernates containing membrane components shed as a result of temperature shift (from 4° to 37°C) were obtained from surface-labeled human PBMC by the method described by Sármay et al. (13). 0.6 ml of PBMC suspension (7.5×10^7 cells/ml in serum-free Parker medium TC-199), previously surface-labeled by the lactoperoxidase technique (16) at room temperature, then washed and kept at 4°C, was incubated at 37°C for 60 min in the presence of 50 μg/ml active site-inhibited Tgase, 50 μg/ml active enzyme, or 0.1 μg/ml Ca^{2+}-ionophore A23187. After centrifugation, the protein concentration (Bio-Rad Protein Assay, Bio-Rad Laboratories, Rockville Centre, N.Y.), as well as the concentration of β_2-m (Phadebas β_2-m RIA kit, Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N.J.), was determined in the supernate. Then, portions of the supernates (equal to 1.0 mg protein in each case) were mixed with 0.3-ml packed gel slurries of Sephadex anti-β_2-m immunoadsorbent (3×10^5 particles/ml; Pharmacia) which is previously washed in 10 mM Tris-HCl, pH 8.2 + 0.1 M NaCl + 1.5 mM EDTA. After 2 h of stirring at 37°C, the samples were further shaken at 4°C overnight. Afterwards, the slurries were centrifuged and washed free of unbound materials with the Tris-saline buffer. Specifically bound proteins were released by being boiled for 3 min in 100 μl of SDS-PAGE sample buffer containing 2% SDS and 2% 2-mercaptoethanol, then analyzed by SDS-PAGE. Essentially the same procedure was repeated when the Tgase-catalyzed incorporation of [^{14}C]methylamine (final concentration: 1 mM) into the β_2-m of shedding but noniodianated PBMC was studied. After electrophoresis, the gels were stained and sliced into 1-mm thick pieces; then radioactive measurements were performed (8).

Patching of β_2-m on PBMC

Freshly prepared PBMC suspension was distributed into a series of plastic tubes (2×10^6 cells/tube) in a volume of 200 μl of medium TC-199. In 100 μl of medium, various amounts of Tgase, active site-inhibited Tgase, Ca^{2+}-ionophore A23187, respectively, or medium alone, were added to the cell suspensions, which were incubated for 15 min in a water bath at 37° C afterward. Then, the following steps were performed: (*a*) pelleting of the cells and washing them twice with medium TC-199 at room temperature; (*b*) resuspending each in a 50-μl solution of diluted anti-human β_2-antibody and incubating for 30 min at room temperature; (*c*) washing them twice in ice-cold medium TC-199; (*d*) resuspending each in goat anti-rabbit IgG antibody labeled with fluoresceine and incubating in an ice bath for 30 min; (*e*) washing them twice in ice-cold medium TC-199; and (*f*) fixing them in 1% paraformaldehyde diluted in phosphate-buffered saline. (In two experiments, the cells were first treated according to step (*b*), and only then with 5 μg of Tgase; the following steps were the same.) After a last washing in medium TC-199, the cell pellet was resuspended in buffered glycerol and observed under a fluorescence microscope (Fluoval, Zeiss). The proportion of cells showing spots or patches was inferred from examination of 150–200 cells/sample.

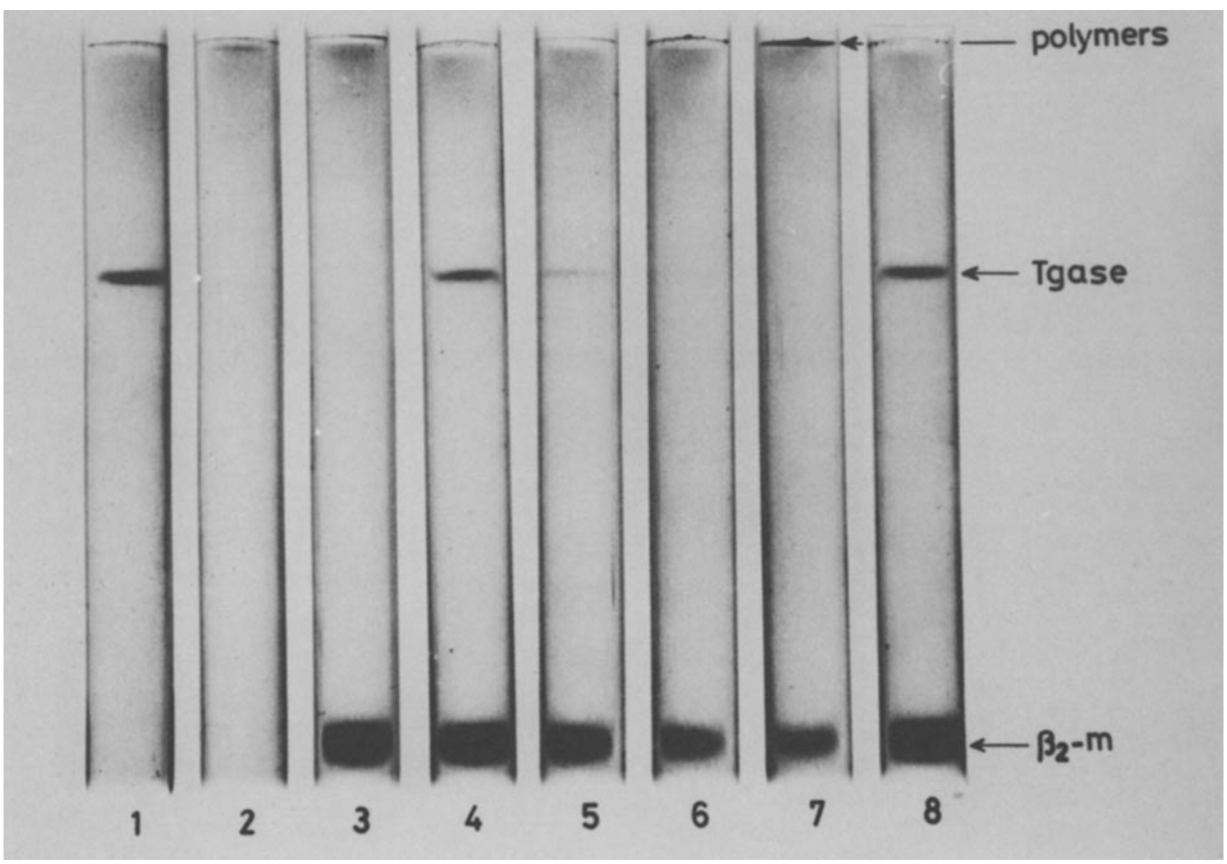

FIGURE 1 Crosslinking of β_2-m by tissue Tgase. Proteins were separated on 10% polyacrylamide SDS gels after incubation at 37°C and subsequent denaturation. *1:* Guinea pig liver Tgase incubated for 60 min without Ca^{2+}. *2:* Tgase incubated for 60 min in the presence of Ca^{2+}. *3:* β_2-m. *4:* β_2-m and Tgase incubated for 60 min without Ca^{2+}. *5–7:* β_2-m and Tgase incubated for 10, 30, and 60 min, respectively, in the presence of Ca^{2+}. *8:* β_2-m and Tgase incubated for 60 min in the presence of Ca^{2+} and 30 mM [^{14}C]methylamine (83,500 cpm/nmol); 25.2 μg of β_2-m was run on the gel; and the cpm measured in the β_2-m band was 179,825.

RESULTS

The appearance of high-molecular-weight polymers on the top of 10% SDS polyacrylamide gels with a concomitant decrease of β_2-m was observed when samples obtained from the Ca^{2+}-containing incubation mixture of β_2-m and Tgase were electrophoresed (Fig. 1). The reaction was time-dependent and accompanied by the disappearance of Tgase monomers as well. Although the self-polymerization (1) of the enzyme was complete within 60 min, there were still β_2-m monomer molecules left. The latter could not be polymerized, even by the addition of more enzyme (results not included in the figure). When 30 mM [^{14}C]methylamine was also included in the incubation mixture, the polymerization of β_2-m did not take place (Fig. 1, gel 8). However, there was an incorporation of labeled methylamine into β_2-m. From the measured radioactivity incorporated into the known amount of β_2-m run on the gel, the number of methylamine molecules coupled to β_2-m was estimated to be an average of one methylamine molecule per β_2-m monomer.

β_2-m, like several other proteins, is shed into the media of PBMC when the cells are exposed to a temperature shift (13). Under our experimental conditions, the protein and β_2-m concentrations in the sheddings of PBMC varied between 2.0 and 2.2 mg/ml and 50 and 80 μg/ml, respectively. When the radioactive proteins shed from iodinated PBMC in the presence of Tgase and bound to anti-β_2-m immunoadsorbent were analyzed on SDS-PAGE, a 33,000, a 70,000, and a higher (not entering into 10% gel) molecular-weight protein were found in addition to the 45,000 (human leukocyte antigen [HLA]) and 12,000 (β_2-m) molecular weight proteins found in the control sample (shed in the presence of active site inhibited enzyme) (Fig. 2). In the case of Ca^{2+}-ionophore A23187, the two additional peaks were also detected (33,000 and 70,000 mol wt).

In a separate experiment, noniodinated PBMC were subjected to shedding in the presence of 1.0 mM [^{14}C]methylamine and either Tgase or Ca^{2+}-ionophore A23187. β_2-m in the shed supernates was bound to anti-β_2-immunoadsorbent and then run on SDS-PAGE in the presence of internal standard molecular-weight proteins. The following values in counts per minute (cpm) were measured in the band corresponding to β_2-m: 85 (shedding in the presence of active site inhibited enzyme), 19,221 (active enzyme), and 3,152 (Ca^{2+}-ionophore A23187), showing the apparent incorporation of [^{14}C]methylamine into β_2-m of the shedding PBMC by the enzyme.

β_2-m can not be patched or capped on the cell surface by adding only anti-β_2-m antibodies to the cells, not even when a second antibody (directed against the anti-β_2-m antibody) is added to the anti-β_2-m antibody-coated cells in the cold (12). This was confirmed in our experiments with PBMC (Fig. 3 *a*). However, when PBMC were incubated with Tgase at 37°C for 15 min before the coating procedure, the fluorescence, originating from the fluoresceine-labeled second antibody, was not found to be homogeneously distributed on the cell surface but was concentrated in spots and patches of various sizes (Fig. 3 *b*, *c*, and *d*). Fig. 4 shows the increase in the percentage of spotted/patched cells in suspensions treated with varying enzyme concentrations and with Ca^{2+}-ionophore. When the enzyme was added to anti-β_2-m antibody-coated cells, the portion of spotted/patched cells was even less than in the control experiment. The addition of active site-inhibited Tgase did not result in any change in the homogeneous pattern of fluorescence on the cell surface.

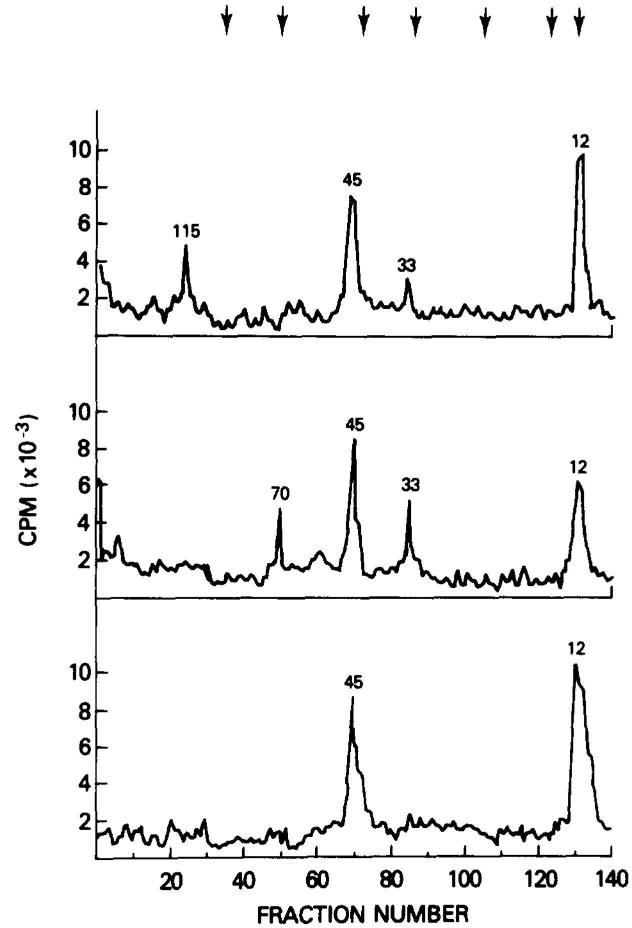

FIGURE 2 SDS-PAGE on 10% gels of radioactive proteins of ^{125}I-labeled PBMC shedding in the presence of active site inhibited Tgase (bottom panel), Tgase (center panel), or Ca^{2+}-ionophore A23187 (top panel) and bound to anti-β_2-m immunoadsorbent. The numbers at the peaks when multiplied by 10^3 indicate apparent molecular weights. The arrows represent the positions of internal molecular weight standards, which from left to right are phosphorilase B (94,000), BSA (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), lysozyme (14,300), and β_2-m.

DISCUSSION

The results indicate that β_2-m is a substrate of Tgase. The result is not surprising because the number of glutamine and lysine residues in the primary amino acid sequence of β_2-m is considered to be exceptionally high (6). However, not all of the β_2-m monomers were converted to multimers by the action of the enzyme on the purified protein. Similarly, there were always β_2 monomers left in the supernate of surface-labeled PBMC shed in the presence of Tgase. In addition, although high concentrations of the enzyme resulted in a substantial increase in the number of spotted/patched cells after Tgase treatment in the antibody-coating experiments, not all cells were affected.

There are several possible explanations for these results. First, the glutamine substrate specificity of Tgase is known to be determined by the amino acid neighbors of the glutamine residue in the polypeptide chain (9). Our observation, that an average of only one methylamine molecule was incorporated

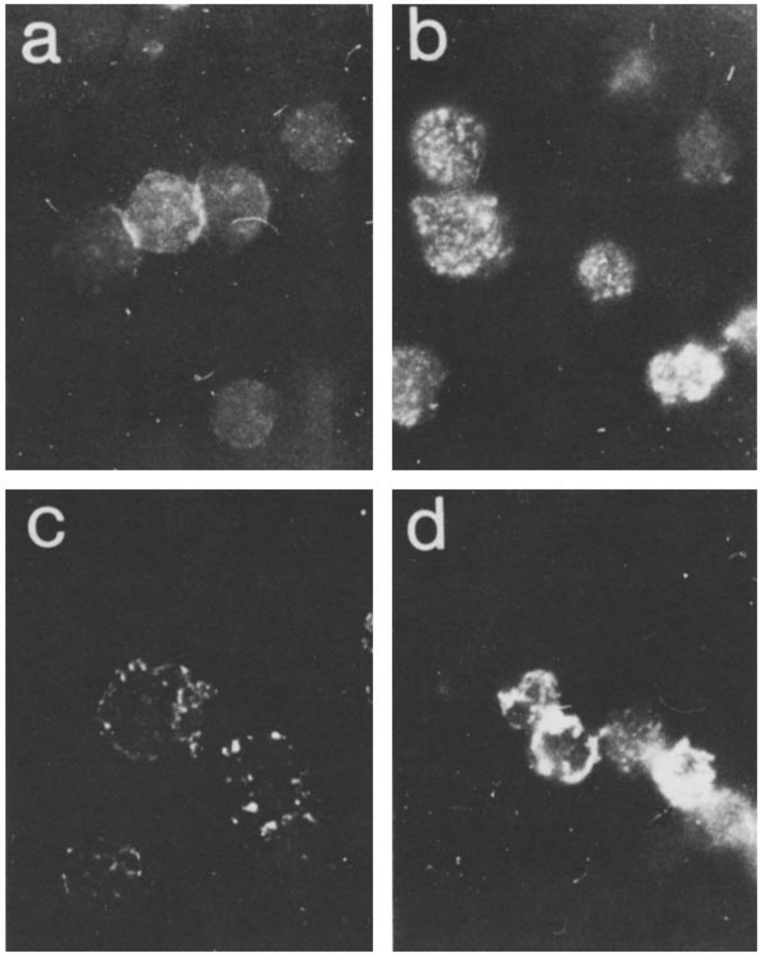

FIGURE 3 Typical pattern of fluorescence distribution on PBMC after the addition of rabbit anti-human β_2-m antibody followed by fluorescein-labeled goat anit-rabbit immunoglobulin. *a*, control; *b*, *c*, and *d*, Tgase-treated cells. There has never been a reaction when only the fluoresceine-labeled second antibody was applied (not shown).

per β_2-m monomer in the isotope incorporation experiment, suggests the presence of few (perhaps only one) appropriate glutamine residue(s) for the enzyme in the sequence. Also, deamination of β_2-m may occur during the preparation of the protein or in vivo (there have been reports showing isomeric forms of β_2-m [3, 14]), and eliminate the appropriate glutamine residue(s) of β_2-m from some of the molecules. Second, the conformation of β_2-m (5) may allow the Tgase-catalyzed formation of intramolecular ϵ-(γ-glutamyl) lysine crosslinks. A recent study (4) depicts the tertiary structure of β_2-m as having two β-sheets on top of each other kept together in a sandwich-like manner by hydrophobic bonds. On the edges of the β sheets one finds the sequences Glu-Pro-Lys and Lys-Ile-Gln. These sequences are so located that internal isopeptide bonds may be easily formed by Tgase, which would prevent the molecule from participating in the polymerization process. Third, various β_2-m "populations" (weakly or strongly attached, complexed to HLA, diffusely packed, etc.) may differ in their availability to interact with the enzyme and other macromolecules on the cell surface.

The catalytic effect of Tgase on β_2-m may be explained in different ways, which do not necessarily exclude one another. (*a*) The polymers formed may consist of only crosslinked β_2-m. On several occasions, we could see the transient appearance of a faint band of β_2-m dimer on the gel. Its disappearance and our unsuccessful attempts to find other products of the polymerization process can be explained by assuming that the dimer (or trimer, etc.) form is just as good a substrate to the enzyme as the monomeric one. (*b*) β_2-m may be copolymerized with Tgase, although it should be noted that changing the β_2-m:enzyme ratio in separate experiments did not alter the final portion of polymerized β_2-m (data not shown in figures). (*c*) In the cellular experiments, β_2-m may be crosslinked to other membrane protein(s). The presence of newly formed anti-β_2-m antibody-reactive proteins other than β_2-m and HLA antigen in the supernate of PBMC shed as a result of Tgase action seems to support this possibility. (*d*) The change in the distribution of β_2-m on the surface of PBMC may be the result of the enzyme-catalyzed polymerization of another protein which is closely associated with β_2-m. We have shown that several proteins (besides β_2-m) can be labeled on the cell surface using Tgase and a radioactive amine (8).

Both β_2-m and Tgase are widely distributed and have already been implicated (in some cases with little evidence) in a number of biological functions. At present, it would be premature to speculate on the interrelationship of the two proteins in these functions. Nevertheless, our finding, that Ca^{2+}-ionophore A23187 activating Tgase in PBMC led to crosslinkage of

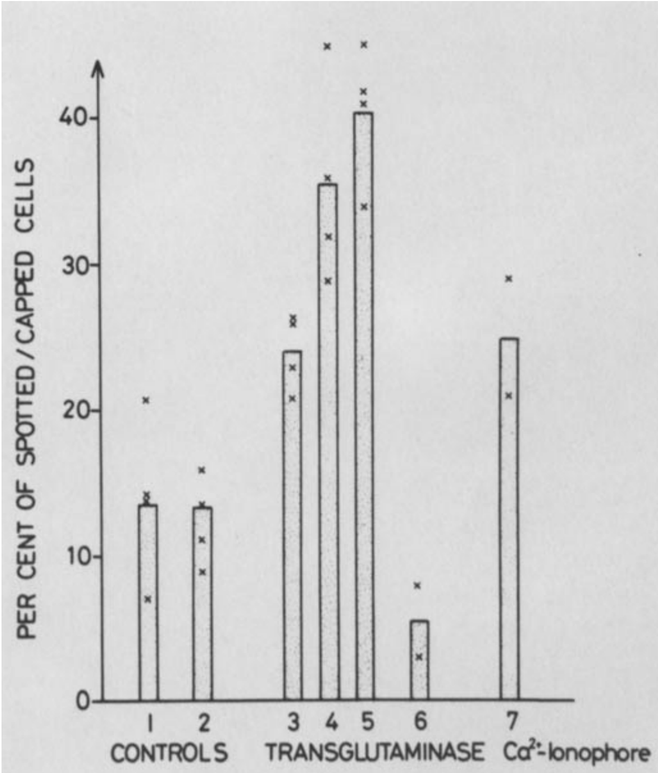

FIGURE 4 The percentage of cells showing spots or patches in differently treated suspensions of PBMC after the addition of rabbit anti-human β_2-m antibody, followed by fluoresceine-labeled goat anti-rabbit immunoglobulin. 2×10^6 cells/tube was incubated at 37°C for 15 min with medium TC-199 (*1*), 25 μg of active site-inhibited Tgase (*2*), 2.5 μg of Tgase (*3*), 5 μg Tgase (*4*), 25 μg of Tgase (*5*), or 0.03 μg of Ca^{2+}-ionophore A23187 (*7*), respectively, before the addition of the antibodies. In the case of *6*, the incubation of PBMC with Tgase took place after the addition of anti-human β_2-m and subsequent washing.

cellular proteins (L. Fésüs, unpublished observations), and affected the shedding and patching of the cells, similar to the outside addition of Tgase, suggests that intracellular Tgase activated during various cellular phenomena can also use β_2-m as a substrate.

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