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TUNICAMYCIN TREATED FIBROBLASTS SECRETE A CATHEPSIN B-LIKE PROTEASE

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We have investigated the effect of tunicamycin on the localization of lysosomal hydrolases in chicken embryo fibroblast cultures. We showed that treatment with tunicamycin (0.05 μg/ml) resulted in a 7-10 fold increase in the cathepsin B-like activity in the culture medium compared to untreated cultures. The protease activity was identified as cathepsin B-like based on 1) substrate specificity (benzoylpro-phe-arg[^{14}C]anilide is rapidly hydrolyzed), 2) the pH optimum for activity of 5.5, 3) inhibition by thiol reactive compounds, 4) inhibition of the activity by leupeptin but not by pepstatin or phenylmethylsulfonyl fluoride, and 5) by the demonstration of a protease with similar properties in the lysosomal fraction of untreated cultures. The secretion of the cathepsin B-like protease was specific and not due to leakage from damaged cells.

INTRODUCTION:

Sly and coworkers have proposed a model in which lysosomal enzymes rely on a unique terminal phosphomannosyl recognition marker to be segregated from secretory products following synthesis on the rough endoplasmic reticulum (for recent reviews see 1,2). Based on this model it can be expected that newly synthesized lysosomal hydrolases, lacking the recognition marker, will not be segregated from secretory products, hence (i) will be exported from the cell and (ii) will not be delivered to lysosomes. Treatment of cells with TM, an inhibitor of glycosylation of N-asparagine linked oligosaccharides (3,4), should prevent synthesis of the carbohydrate recognition marker on lysosomal hydrolases. Cells treated with TM frequently have reduced levels of membrane and secretory glycoproteins, and, in several instances, the reduction in specific glycoproteins can be directly correlated with enhanced proteolytic degradation (for recent reviews see 5,6). Misrouting and packaging of lysosomal proteases into secretory vesicles may explain, in part, the enhanced

ABBREVIATIONS: TM, tunicamycin; PMSF, phenylmethylsulfonyl fluoride.

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proteolysis of many glycoproteins in TM-treated cells. In the present report we demonstrate, for the first time, that TM-treated fibroblasts secrete a cathepsin B-like protease.

MATERIALS AND METHODS:

TM was a gift from Dr. Gakuzo Tamura via the Drug Evaluation Branch of the National Cancer Institute. The radiochemicals were purchased from New England Nuclear, Boston, MA.; leupeptin, pepstatin, cycloheximide, puromycin, colchicine, vinblastine, PMSF, and polyvinylpyrrolidone (PVP-40) from Sigma Chemical Company, St. Louis, MO.

Cultures of chicken embryo fibroblasts were prepared and maintained as previously described (12). For the protease secretion experiments, serum-containing medium was aspirated, the cell culture washed once with Eagle's Basal Medium containing 0.05% (W/V) polyvinylpyrrolidone and incubated in this medium with or without TM (0.05 μg/ml). The serum-free medium was collected at designated intervals, centrifuged at 10,000 x G for 10 min at 5°C to remove cells or debris, and the supernatants were stored at -20°C.

To measure intracellular enzyme levels, cells were detached from the dish by scraping with a rubber policeman, collected by centrifugation, and washed once with Hank's balanced salt solution without Ca^{++} or Mg^{++}. The cells were then suspended in 0.5 ml of 50 mM Tris-HCl, pH 7.0 at 5°C, frozen and thawed once, sonicated by 3 bursts of 10 sec each in a cold bath (Ultrasonic Cleaner, Laboratory Supplies Co., Hicksville, N.Y.) and mixed with 2 μl of 20% Triton X-100. After 10 min on ice, the samples were clarified by centrifugation for 1 h at 18,000 rpm in Sorvall rotor SM 24. The supernatant solutions were termed cell extracts. To obtain subcellular fractions, cells suspended in 0.25 M sucrose, were disrupted by 20 strokes in a Potter-Elvehjen homogenizer at 5°C and fractionated on colloidal silica gradients as described by Rome et al. (7).

Protease activity was measured by incubating the following reaction mixture at 37°C for 3 h: 50 μl of medium or cell extract (containing 10-50 μg protein), 50 μl of 100 mM sodium acetate buffer (pH 5.5), and 5 μl (22.4 μM final concentration) of substrate (Benzoylpro-phe-arg [^{14}C]-anilide). Cathepsin B cleaves between the arginine and anilide thereby releasing free [^{14}C]-anilide. Blanks contained fresh medium, buffer or heat-treated (100°C for 5 min) cell extract. The reaction, which is linear for at least 3 h, was terminated by heating in a 100°C water bath for 1 min. Following neutralization with borate buffer (100 μl of 200 mM, pH 9.0), [^{14}C]-anilide was quantitatively extracted by adding 250 μl of hexane, mixing vigorously and collecting the organic layer after phase separation. Samples rich in protein were centrifuged 10,000 x G for 10 min to get phase separation. The extraction was repeated 3 times and the organic phases were pooled, diluted into Econofluor (New England Nuclear) and counted by liquid scintillation spectrometry. Protease activity in the medium was stable at -20°C for more than a month.

Lactate dehydrogenase (EC 1.1.1.27) was measured as described by Wroblewski and Ladue (8), β-hexosaminidase (EC 3.2.1.30) as described by Hall et al. (9) and monamine oxidase according to the procedure of Wurtman and Axelrod (10). Protein determinations were made by the phenol method (11).

RESULTS:

TM treatment increased the level of cathepsin B-like protein activity in the medium by 7 to 10 fold compared to control cultures (Fig 1). TM at a concentration of 0.05 μg/ml inhibited [^{3}H]-mannose incorporation into trichloroacetic acid-insoluble cellular fraction by more than 90% while it reduced

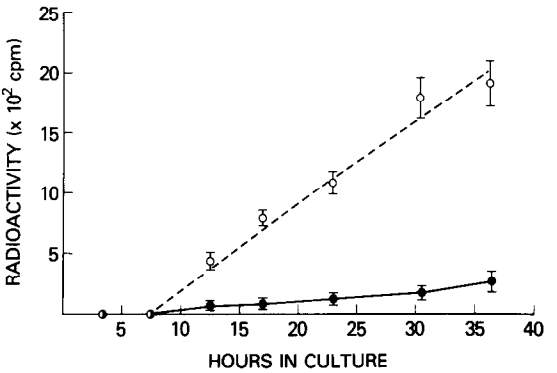

Figure 1: Effect of TM on the levels of cathepsin B-like activity in the medium. Chick embryo fibroblasts were incubated in serum-free medium with or without TM (0.05 μg/ml). Aliquots of the medium were assayed at designated intervals for activity as described in "Materials and Methods." Medium from control cultures (closed circles), medium from TM-treated cultures (open circles).

[^{14}C]-leucine incorporation less than 20% (12, and unpublished data). The 6-8 h lag in the appearance of the protease activity in the medium is correlated with a similar lag in the depletion of [^{3}H]-mannose incorporation following TM addition. The protease activity released into the medium of TM-treated cells did not bind to concanavalin A-Sepharose in contrast to a similar activity present in the homogenate of untreated cultures (results not shown). The specificity of binding was shown by inhibition with α-methyl-D-mannoside. These findings indicate that a nonglycosylated protease, with cathepsin B-like substrate specificity, is secreted by TM-treated fibroblasts.

The protease activity secreted by TM-treated cells had a pH optimum of 5.5 and the activity was inhibited by $HgCl_2$, $AgNO_3$ and iodoacetic acid (thiol protease inhibitors), and also by leupeptin (an arginyl protease inhibitor) (shown in Table 1). Pepstatin (an inhibitor of cathepsin D) and PMSF (a serine protease inhibitor) had no significant effects on the activity. This inhibitor profile is indistinguishable from that of cathepsin B (13,14) and, taken together with the specificity of cleavage of the substrate and the acid pH optimum, strongly suggests that the secreted protease is lysosomal cathepsin B.

A similar cathepsin B-like activity was found to be abundant in lysosomal fractions prepared from homogenates of untreated cells (see Fig. 2 and

Table 1: Effect of Potential Inhibitors on Cathepsin B-like Activity

Agent	Concentration (mM)	Activity in Medium % Control	Activity in Cell Extract % Control
None	-	100	100
Iodoacetic Acid	0.1	18	n.d.
$HgCl_2$	0.1	0	27
$AgNO_3$	0.5	30	38
Leupeptin	0.002	0	11
Pepstatin	0.005	100	100
PMSF	1.0	92	85

Medium from TM-treated cultures (30 h incubation) and cell extract from untreated cultures were incubated ± the potential inhibitor for 5 min at 37°C before adding the substrate. The residual activities were measured as described under "Materials and Methods." Values are the means of three determinations.

Table 1), and, as expected, the lysosomal fractions of TM-treated cells contained reduced levels of cathepsin B-like activity. Incubation of cells with TM for 30 h resulted in a 40% decrease in intracellular levels of cathepsin B-like activity compared to control cells. A direct comparison between the levels of intracellular and secreted cathepsin B-like activity was not possible since the high protein content of cell fractions reduced the apparent protease activity in these samples.

The release of protease activity by TM-treated cells appears to be specific and not due to cell injury since lactate dehydrogenase levels in medium were not increased above control levels after 36 h incubation with TM. Also, the release of protease activity by TM-treated cells is strongly inhibited by cycloheximide, puromycin, colchicin, and vinblastine (see Table 2). The latter results suggest that secretion is dependent on protein synthesis and structurally intact microtubules (15).

DISCUSSION:

The results presented indicate that the asparagine-linked carbohydrate moiety of a lysosomal protease, with cathepsin B-like properties, is required for its intracellular retention. This is supported by several findings. First, treatment with TM, an inhibitor of N-glycosylation, promoted the

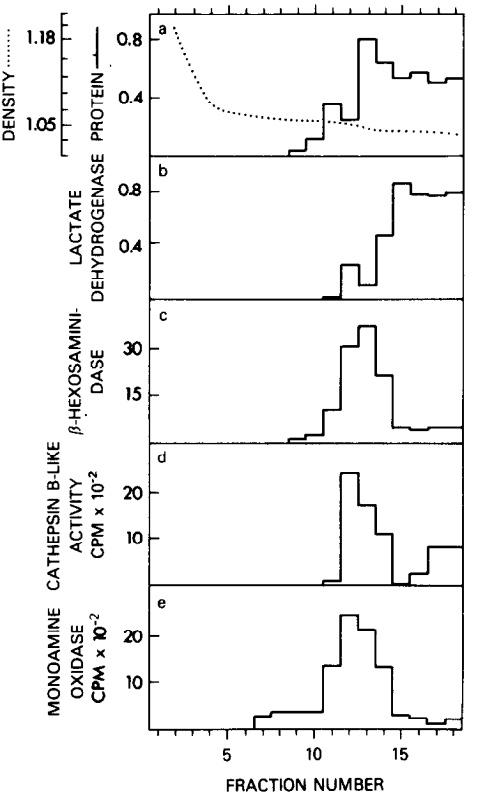

Figure 2: Fractionation of post-nuclear supernatant from fibroblasts on colloidal silica gradient. Cellular fractions were prepared from chicken embryo fibroblasts as described in "Materials and Methods." Marker enzyme activities are expressed as units/ml, density as g/ml and protein as mg/ml.

release of the protease into the medium with a concomitant loss of the activity of a lysosomal-associated protease with similar properties. Second, the cathepsin B-like protease in the medium of TM-treated cells did not bind to

Table 2: Effect of Potential Inhibitors on Export of Cathepsin B-like Activity by Tunicamycin-treated Cells

Agent	Concentration (10^{-6}M)	Activity in Medium % Control
None	-	100
Vinblastine	0.1	20
Colchicine	10.0	3
Puromycin	20.0	0
Cycloheximide	360.0	3

Cultured fibroblasts were incubated in serum-free medium with TM (0.05 μg/ml) for 10 h at 37°C, fresh serum-free medium containing TM ± one of the agents listed above was added to the cultures for an additional 15 h incubation and activity in the medium was measured as described in "Materials and Methods." Values are the means of three determinations.

concanavalin A-Sepharose in contrast to the corresponding protease activity found in the lysosomal fractions prepared from homogenates of untreated cells. The secretion of the acid protease was inhibited by agents which impair microtubule assembly, but the mechanism by which it is "packaged" and transported to the exterior of the cell is currently unknown. We favor the possibility that the newly synthesized cathepsin B-like protease is directly secreted via the route utilized by the "constitutively" secreted proteins such as procollagen.

The protease activity has been identified as cathepsin B-like based upon substrate requirement, acid pH optimum for activity, inactivation by thiol reactive compounds and inhibition by leupeptin but not by pepstatin or PMSF.

Cells treated with TM frequently have reduced levels of membrane and secretory glycoproteins (for recent review see 5,6). Also, increased rates of proteolysis after TM-treatment of cells has been shown for fibronectin (12), hemagglutinin precursor of influenza virus (16), adenocorticotropin-endorphin precursor (17,18) and acetylcholine receptor (19). Leupeptin, a potent inhibitor of cathepsin B activity, has been shown to inhibit rapid degradation of several glycoproteins after TM treatment of cells (19-21). Based on the present findings, it is plausible that the co-localization of cathepsin B-like proteases with secretory and membrane glycoproteins, in intracellular transport vesicles, may explain the decrease and rapid degradation of glycoproteins in TM-treated cells. Reports that (i) the internal pH of secretory vesicles is acidic (22-24), and (ii) that glycosylation-depleted proteins are inherently more sensitive to proteases (25-27) are consistent with this suggestion.

Finally, the data presented here extend and are consistent with the observations made by von Figura et al. (28) that human skin fibroblasts treated with TM secrete the lysosomal enzyme β-N-acetylglucosaminidase with an accompanying depletion in the cell-associated content of this enzyme.

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