Vol. 99, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 16, 1981 Pages 221-227

EVIDENCE FOR THE INVOLVEMENT OF TRANSGLUTAMINASE IN THE UPTAKE OF VITELLOGENIN BY XENOPUS laevis OÖCYTES

Linda M. Tucciarone and Kenneth D. Lanclos*
Department of Cell and Molecular Biology
Medical College of Georgia
Augusta, Georgia 30912

Received January 22, 1981

Summary

The yolk protein, vitellogenin, is sequestered by the developing oöcyte by receptor-mediated endocytosis, the process by which cells bind and internalize extracellular proteins. Endocytosis of a variety of proteins follows a similar pathway, whereby internalization of receptor-bound ligand takes place over clathrin-coated regions of the cell membrane. The protein cross-linking enzyme, transglutaminase, has been reported to be essential for the receptor-mediated endocytosis of insulin and α_2-macroglobulin. In this study, the presence of transglutaminase activity was demonstrated in the Xenopus laevis ovary and was effectively inhibited by poly L-lysine, an inhibitor of vitellogenin uptake, and dansylcadaverine, a known inhibitor of transglutaminase activity. Two other less potent inhibitors of transglutaminase, methylamine and bacitracin produced partial inhibition of the ovarian enzyme. Furthermore, dansylcadaverine and methylamine were found to inhibit the appearance of vitellogenin in the yolk platelets of the oöcyte.

Introduction

Selective sequestration of the yolk protein, vitellogenin, is accomplished in the Xenopus laevis oöcyte via the process of pinocytosis (1). This phenomenon manifests many of the characteristics associated with receptor-mediated endocytosis which has been described as a mechanism for the internalization of many proteins (for review, see Ref. 2). During the peak of greatest endocytotic activity (3), the cell membrane of the oöcyte is studded with coated pits, the site of internalization. These coated pits pinch off to form coated vesicles, an intermediary organelle in the transport of vitellogenin to the yolk platelets (4).

The mechanisms involved in receptor-mediated endocytosis are the subject of much investigation. It appears that there is a step prior to internali-

* Dr. Kenneth D. Lanclos
Department of Cell and Molecular Biology
Medical College of Georgia
Augusta, Georgia 30912

0006-291X/81/050221-07$01.00/0

zation that involves the clustering of receptor-bound ligands to the coated pit regions of the membrane. This has been observed with epidermal growth factor and insulin in human fibroblasts (5). This clustering process is believed to be catalyzed by transglutaminase, an enzyme that forms ε-(γ-glutamyl)lysine crosslinks between protein molecules. This proposal is based upon the observation that alkylamines and certain lysine-containing peptides that inhibit transglutaminase activity also inhibit migration of receptor-bound ligands to the coated pits and thereby inhibit internalization (6). In addition, both the endocytotic process and the transglutaminse enzyme require the presence of extracellular Ca^{+2} ions.

We report here the ability of dansylcadaverine, methylamine and bacitracin, inhibitors of transglutaminase (6), and poly L-lysine, an inhibtor of vitellogenin uptake (7), to inhibit transglutaminase activity in the Xenopus ovary as well as the rat liver. In addition, dansylcadaverine and methylamine were found to inhibit the uptake of vitellogenin by oöcytes.

Materials and Methods

The care, maintenance and bleeding of animals was performed as previously described (8). Synthesis of vitellogenin was induced in female Xenopus laevis by the injection of 2 mg of estradiol-17β on days 1 and 4. On day 8, the toads were bled and vitellogenin was isolated from the serum by molecular sieve chromatography. Vitellogenin eluted in the void volume of a Sephacryl-200 column previously equilibrated with solution O-R2 (9). Vitellogenin was labelled by one of the following 2 ways: either in vivo prior to isolation with [^{32}P]-orthophosphoric acid or in vitro by radioiodination using IODO-GEN technique (10). This latter procedure regularly yielded preparations of approximately 70 x 10^6 cpm/mg.

Female Xenopus laevis were given injections of 1000 units of human chorionic gonadotropin on days 1 and 7. On day 8, the ovaries were removed by laparotomy and stave IV oöcytes (1.0 mm diameter) were manually dissected from their follicles with watchmakers forceps. Groups of 30-40 oöcytes were placed in cell culture plates containing ^{125}I-vitellogenin in O-R2 for 15 hrs at 20°C. In the inhibitor studies oöcytes were preincubated for 1 hr in the presence of dansylcadaverine or methylamine and then transferred to labelled media containing the same inhibitor. Oöcytes were washed with O-R2 containing 1 mg/ml vitellogenin and then homogenized in 15% sucrose containing a HEPES-PVP buffered solution (11). The homogenate was layered over a 19-50% sucrose gradient containing HEPES-PVP buffered solution and centrifuged for 12 hr at 40,000 rpm in an SW41 rotor. The gradients were fractionated, the absorbance monitored and 0.2 ml fraction were collected. Fifty µl samples were assayed for radioactivity by spotting the sample on filter paper, placed in cold 10% TCA, washing and drying in ethanol: acetone (1:1) before counting

The 2,5000 x g supernate from 10% rat liver homogenate was used as the source of enzyme in the transglutaminase assay (12). The reaction mixture

contained 2.2 mg/ml casein, 2.8 mM DTT, 1.2 mM putrescine, 28 μCi [^{3}H]-putrescine (NEN) and 50 mM TRIS, pH 7.5. The reaction was carried out at 37°C in a volume of 200 μl. The Ca^{+2} dependent activity was measured by the addition of 5 mM $CaCl_2$ to one tube and 14 mM EGTA to another tube. For the inhibitor studies the appropriate concentration of dansylcadaverine, methylamine, bacitracin or poly L-lysine were added to the reaction mixture. After a 20 min incubation, 25 μl samples (in triplicate) were spotted on filter paper and processed as described above. Under these conditions, all assays were linear with time and protein concentration.

For the ovarian transglutaminase assay, the same protocol was followed except that the 2500 x g supernatant from a 50% ovarian homogenate was used as the soucre of enzyme and the assay was run at 20°C. The ovary was removed from a Xenopus laevis stimulated with hCG as described above.

Results

The enzymatic activity of transglutaminase (TGase), the formation of ε-(γ-glutamyl)-lysine bonds between casein and [^{3}H]-putrescine, was measured in the rat liver and an hCG-stimulated ovary in the presence and absence of the inhibitors of TGase and an inhibitor of endocytosis of vitellogenin, poly L-lysine. The results of these experiments are shown in Fig. 1. They demonstrate that dansylcadaverine, the most potent inhibitor of liver transglutaminase, is an effective inhibitor of the ovarian enzyme. Methylamine and bacitracin were only able to partially inhibit ovarian TGase activity, with bacitracin being the least effective of the two. Poly L-lysine also proved to be an effective inhibitor of both rat liver and ovarian transglutaminase. In each case, the effects of the inhibitor on the ovarian transglutaminase closely parallels their effect on the rat liver enzyme, a common source of TGase.

To determine if transglutaminase activity is involved in the endocytosis of vitellogenin, oöcytes, pretreated for one hour with either dansylcadaverine or methylamine, were incubated with radioiodinated-vitellogenin and either dansylcadaverine or methylamine. In these experiments, we used ^{125}I vitellogenin iodinated in vitro by the IODO-GEN method, a procedure that is very mild and yields preparations with high specific activities. To demonstrate the suitability of this iodinated vitellogenin as a biological probe for sequestration, the uptake of iodinated vitellogenin was compared to that of vitellogenin labelled in vivo with ^{32}P on a sucrose gradients that shows the flow of labelled vitellogenin from the pinocytotic vesicles to the yolk plate-

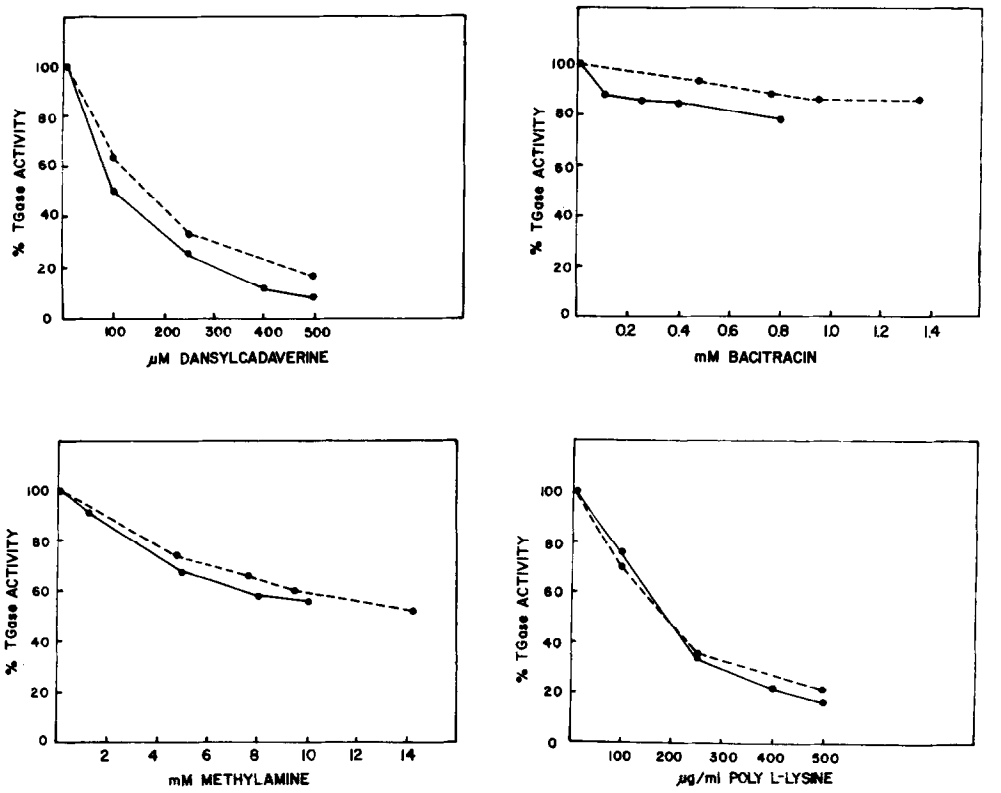

FIGURE 1. Inhibition of TGase activity. TGase activity was assayed by the incorporation of [^{3}H] putrescine into casein. Rat liver extracts (●——●) or ovary extracts (●---●) were incubated in the presence of the indicated concentrations of inhibitors for 20' before applying to paper filters for processing. 100% TGase activity represents [^{3}H] putrescine incorporated in absence of any inhibitors.

lets. The labelling pattern of the oöcyte subcellular organelles is shown in Fig. 2, in which ^{125}I-vitellogenin was used as the substrate. The pattern is identical when [^{32}P]-vitellogenin is used as the substrate (data not shown).

We measured the effect of dansylcadaverine and methylamine on the uptake of ^{125}I-vitellogenin by determining the amount of incorporation of ^{125}I-vitellogenin into the yolk platelets by measuring the area under the curve. After the oocytes were incubated for 15 hrs, in the presence of ^{125}I-vitellogenin alone, the majority of the acid-insoluble radioactivity was found associated with the light and heavy yolk platelets (Fig. 2, fractions 47-52 and 53-58, respectively). In addition there is a small peak of radioactivity, representing 9.5% of the

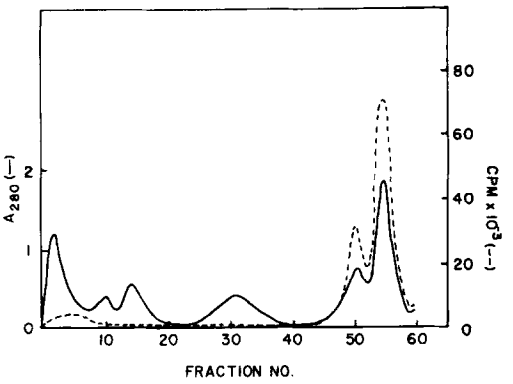

FIGURE 2. Sucrose gradient analysis of the uptake of ^{125}I-vitellogenin by the oöcyte. Oöcytes, incubated for 15 hrs in the presence of ^{125}I-vitellogenin, were rinsed, homogenized, and layered over 19-50% sucrose gradients, and centrifuged at 196,000 x g for 12 hrs.

total radioactivity incorporated, that is found associated with the membrane vesicle-containing fraction of the gradient (Fig. 2, fractions 1-8). Incubation of the oöcytes with ^{125}I-vitellogenin plus dansylcadaverine or methylamine, led to a decrease of acid-insoluble radioactivity associated with the yolk platelets peaks at the bottom of the gradient. These results are given in Table I. At a concentration of 100 µM dansylcadaverine, there was a 17% decrease in the amount of radioactivity associated with the yolk platelets. At

Table I

Effect of dansylcadaverine and methylamine on vitellogenin incorporation into subcellular fractions of Xenopus laevis oöcytes

Inhibitor	% of control Fractions 47-58
100 µM dansylcadaverine	83
200 µM dansylcadaverine	43
15 mM methylamine	71

Oocytes were incubated with ^{125}I-vitellogenin in the presence of the inhibitor for 15 hr. following preincubation with the inhibitory alone. Cells were washed, homogenized and centrifuged on 19-50% sucrose gradients. Gradients were fractionated and 50 µl samples were precipitated with TCA on paper filters and counted. Controls represent uptake into oocytes incubated with ^{125}I-vitellogenin in absence of any inhibitor.

200 μM dansylcadaverine, there was a 54% decrease in incorporation. Incubation with 15 mM methylamine led to a diminution of 28% in the yolk platelets.

Discussion

These results demonstrate that there is present in the gonadotropin stimulated ovary an enzymatic activity that has the properties of transglutaminase, namely Ca^{+2}-dependent activity, a sensitivity to alkylamines, bacitracin and, as we have demonstrated, poly L-lysine. The following characteristics of transglutaminase have also been observed: 1) L-lysine containing peptides inhibit TGase activity in NRK cells (12); and 2) the amine binding site of TGase preferentially accommodates peptide bound L-lysine residues (14). These data together with the finding that poly L-lysine markedly inhibits vitellogenin uptake (7) suggests that a transglutaminase activity may be involved in the specific sequestration of vitellogenin by oöcytes. Indeed, we have shown that dansylcadaverine and methylamine have the effect of decreasing the incorporation of radio-labelled vitellogenin into the yolk platelets. In addition, we observed that incubation with dansylcadaverine also results in a decrease in radioactivity associated with the vesicle containing fraction of the oöcytes. Since the pinocytotic vesicles are the organelles of intracellular transport to the yolk platelets, having derived from the cell surface, we have concluded that dansylcadaverine is inhibiting the uptake of vitellogenin.

These observations closely parallel those seen in the case of receptor-mediated endocytosis of α_2-macroglobulin and certain polypeptide hormones (4,11). Dansylcadaverine has been shown to inhibit the internalization of ^{125}I-epidermal growth factor in 3T3 cells (15). Inhibition of transglutaminase activity appears to interfere with internalization by inhibiting a step prior to uptake. This step involves the migration of receptor-bound ligands into the coated pit regions of the cell membrane. The authors have proposed a model whereby the presence of a Ca^{+2}-activated transglutaminase would lead to a crosslinking of proteins in the ligand-receptor complexes

clustered in the coated pit, the result of which would be to make this aggregation irreversible. Therefore, it is possible that a similar step of clustering of receptor-bound vitellogenin is occurring in the oöcyte prior to its internalization.

Acknowledgements

This investigation was supported by the National Institutes of Health Grant BRS-RR-05365.

References

1. Wallace, R.A. and Dumont, J.N. (1968) Journ. Cell. Phys. 72, 73-89.
2. Goldstein, J.L., Anderson, R.W. and Brown, M.S. (1979) Nature 279, 679-685.
3. Wallace, R.A., Jared, D.W. and Nelson, M.W. (1979) Journ. Exp. Zool., 175, 259-270.
4. Dumont, J.N. (1972) Journ. Morpho. 136, 153-180.
5. Maxfield, F.R., Schlesinger, J., Schecter, Y., Pastan, I. and Willingham, M.C. (1978) Cell 14, 805-810.
6. Davies, P.J.A., Davies, D.R., Levitzki, A., Maxfield, F.R., Milhaud, P., Willingham, M.C. and Pastan, I.H. (1980) Nature 283, 162-167.
7. Wallace, R.A. and Ho, T. (1972) Journ. Exp. Zool., 187, 303-318.
8. Wallace, R.A. and Jared, D.W. (1968) Can. Journ. Biochem., 46, 953-959.
9. Wallace, R.A., Jared, D.W., Dumont, J.N. and Sega, M.W. (1973) Journ. Exp. Zool., 184, 321-334.
10. Fraker, P.J. and Speck, C.J., Jr. (1978) Biophys. Biochem. Res. Commun., 80, 849-857.
11. Jared, D.W., Dumont, J.N. and Wallace, R.A. (1973) Dev. Biol. 35, 19-28.
12. Lorand, L., Campbell-Wilkes, L.K. and Cooperstein, L. (1972) Analyt. Biochem. 50, 623-631.
13. Levitzki, A., Willingham, M. and Pastan, I. (1980) Proc. Natl. Acad. Sci., 77, 2706-2710.
14. Folk, J.E. and Finlayson, J.S. (1977) Adv. Prot. Chem., 31, 1-33.
15. Haigler, H.T., Maxfield, F.R., Willingham, M.C. and Pastan, I. (1980) Journ. Biol. Chem. 255, 1239-1241.