Transmembrane delivery of insulin into cells through α_2-macroglobulin receptor-mediated endocytosis

Fumiaki Ito and Nobuyoshi Shimizu

Department of Cellular and Developmental Biology, University of Arizona, Tucson, AZ 85721, USA

Received 14 December 1982

Endocytosis *Hormone receptor* *Insulin* *α2-Macroglobulin* *Percoll gradient* *Lysosomes*

1. INTRODUCTION

It is known that the primary event of insulin action is the binding of insulin to a specific cell surface receptor [1]. Following binding, insulin complexed with its receptor is aggregated into patches and internalized by endocytosis [2,3]. Eventually, the endocytotic vesicles fuse with lysosomes where insulin is degraded. Whether this insulin processing is necessary for insulin action and if so exactly how it leads to the stimulation of cellular metabolism remains unknown. There have been suggestions that the interaction of insulin with its receptor somehow generates a second, intracellular messenger [4–6]. Yet, despite an extensive search, a messenger which can account for all of insulin's diverse activities has not been identified.

As a step toward understanding the functional significance of the internalized insulin we devised a delivery system of insulin into cells bypassing the insulin receptor. In this paper, we describe the synthesis of a hybrid molecule in which insulin is cross-linked to a human serum protein, α_2-macroglobulin (α2M). Evidence is presented to show that insulin is internalized through α2M receptor-mediated endocytosis and delivered to lysosome-like dense organelles in the form of conjugate.

2. MATERIALS AND METHODS

Mouse Swiss/3T3 cells and CI-3 cells were grown in Dulbecco's modified Eagle medium (DME-medium) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin as described before [7].

α_2-Macroglobulin (α2M) was prepared from human plasma according to the published method [8]. α2M was iodinated by the chloramine-T method [9]. The specific activity of [^{125}I]α2M was 0.7 μCi/μg.

Cross-linking of [^{125}I]insulin with α2M was performed by the method using cystamine [10]. Briefly, porcine insulin (Eli Lilly, 10 mg) was dissolved in 5 ml of 0.01 N HCl and cystamine dihydrochloride (Sigma, 1.13 g) was added. 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide HCl (EDAC, 3.83 mg) was added and the pH was maintained at 4.7 for 10 min with gentle stirring. The reaction was stopped by adjusting the pH to 8.8 with 10 N NaOH and 2 ml of reaction mixture were applied onto a column (1.6 × 18 cm) of Biogel P4, which was equilibrated with 10 mM N-Tris (hydroxymethyl) methyl-2-aminomethane sulfonic acid, pH 8.8, to remove unreacted cystamine dihydrochloride and EDAC. The purified cystaminyl insulin (11.2 μg) was reacted with 1 mCi of ^{125}I using chloramine-T (0.16 μg) in a total reaction volume of 30 μl. After 2 min at room temperature, tyrosine (8 μg) was added to stop further iodination and the reaction mixture was kept overnight at 4°C. The mixture was then incubated with α2M (200 μg) for 2 h at 4°C to allow formation of spontaneous disulfide bonds. Molar ratio of insulin/α2M was 7. The reaction mixture was then applied on a column of Sephadex G-100 equilibrated with 30 mM Na-phosphate buffer (pH 7.5) containing 0.1 M NaCl. A radioactivi-

Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

ty peak which eluted at the void volume was collected. The specific activity of [^{125}I]insulin–α2M conjugate was 0.5 μCi/μg protein.

Binding assay was carried out as described before [7]. Briefly, cells grown to confluency in 35-mm tissue culture dishes were washed twice with 1 ml of binding buffer, consisting of 100 mM Hepes at pH 7.8, 100 mM NaCl, 1.2 mM $MgSO_4$, 5 mM KCl, 10 mM glucose, 1 mM EDTA, 15 mM sodium acetate, and 0.1% bovine serum albumin (BSA, Sigma, RIA grade). Then, the cells were incubated with iodinated α2M (2 nM), insulin (0.27 nM) or conjugate (1.6 nM) at 23°C for the appropriate time. The cells were placed on ice and rinsed 3 times with ice-cold binding buffer. The cells were solubilized in 0.5 N NaOH and the radioactivity was measured in a Beckman 4000 gamma counter. To determine nonspecific binding, unlabelled α2M (500 nM), insulin (3 μM) or both of these ligands was added before the addition of the radioactive ligands. Nonspecific binding was subtracted from total binding to get specific binding.

Percoll density gradient fractionation was performed as previously described [11]. Binding of [^{125}I]conjugate to the confluent cells in 100-mm dishes was carried out at 23°C as detailed above. After 90 min incubation, cultures were washed 3 times with binding buffer. Some cultures were further incubated at 37°C in 8 ml of DME-medium containing 10% FCS for 30 min and rinsed twice with binding buffer. The cells were scraped from the dish in 10 ml of 0.25 M sucrose containing 1 mM EDTA, 10 mM acetic acid and 10 mM triethanolamine, pH 7.4 (SEAT buffer), and lysed by gentle pipetting. Nuclei and unbroken cells were pelleted by centrifugation at 800 rev./min for 10 min and the post-nuclear supernatant (0.9 ml) was layered on a 9 ml suspension of 20% Percoll in SEAT buffer. A gradient was formed by centrifugation at 16000 rev./min for 90 min with an SS34 rotor in a Sorvall RC-5B centrifuge. The gradient was displaced from the bottom. The density of the fractions was determined using an Abbe refractometer.

Marker enzyme assays were performed as described before [11]. These included UDP-galactose-glycoprotein galactosyl transferase (EC 2.4.1.38) for Golgi, acid phosphatase (EC 3.1.3.2) and β-galactosidase (EC 3.2.1.23) for lysosomes and NADPH cytochrome *c* reductase (EC 1.6.2.4) for endoplasmic reticulum.

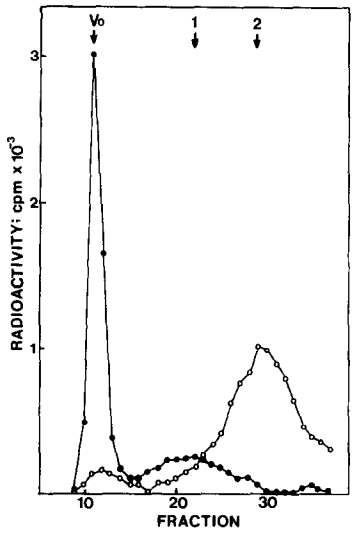

Fig.1. Analysis of [^{125}I]insulin–α2M conjugate by Sephadex G-75 column. Conjugates were treated with 10 mM dithiothreitol (○) or not (●) and subjected to gel filtration on Sephadex G-75 equilibrated with 4 M urea, 1 M acetic acid and 0.1% Triton X-100. Intact and reduced insulin were eluted at positions 1 and 2, respectively. V_0, void volume.

3. RESULTS

[^{125}I]Insulin was cross-linked to α2M by the method using cystamine as described in section 2. The conjugate was analyzed by Sephadex G-75 column chromatography (fig.1). It is seen that a major radioactivity peak was eluted at the void volume, indicating the formation of high molecular weight conjugates consisting of [^{125}I]insulin (M_r 6000) and α2M (M_r 725000). When the conjugate was treated with 10 mM dithiothreitol and analyzed on the same column, the major radioactivity peak shifted to the position at which α- and β-chains of insulin (M_r 3000) elute (indicated as 2). These data clearly show that [^{125}I]insulin was cross-linked to α2M through a disulfide bond. It was calculated that each α2M molecule carried 2–3 insulin moieties.

We tested the binding of [^{125}I]insulin–α2M conjugate to Swiss/3T3 cells and to a variant cell line, CI-3 [7]. The CI-3 cells possess only 10% of the parental Swiss/3T3 cell's insulin binding ability while they retain normal binding ability for α2M (fig.2).

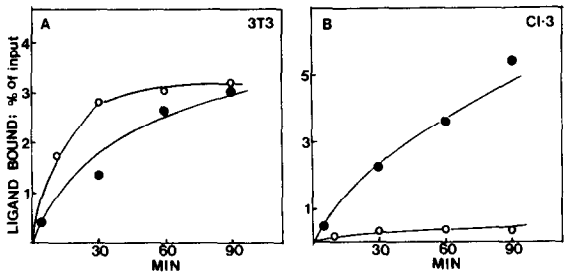

Fig.2. Time course of [^{125}I]α2M and insulin binding to Swiss/3T3 and CI-3 cells. The confluent cultures were incubated with 2 nM [^{125}I]α2M (●), or 0.27 nM [^{125}I]insulin (○) at 23°C. Specific binding was determined by subtracting the binding in the presence of excess unlabelled α2M or insulin from the total binding.

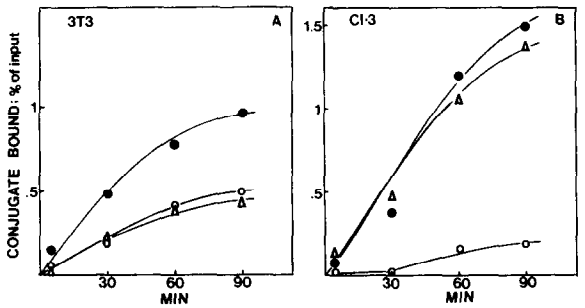

Fig.3. Time course of binding of [^{125}I]insulin–α2M conjugate to Swiss/3T3 and CI-3 cells. The confluent cultures were incubated at 23°C with 1.6 nM conjugate (●), in the presence of excess α2M (○) or insulin (△). Nonspecific binding was determined in the presence of excess insulin and α2M, and subtracted from the total binding.

The binding kinetics of the insulin-α2M conjugate at 23°C are shown in fig.3. Binding could have taken place through two kinds of receptors: one for insulin and the other for α2M. In fact, in Swiss/3T3 cells the addition of insulin or α2M in excess decreased the binding of the insulin–α2M conjugate to 50% and 44% of the control, respectively. In CI-3 cells, the addition of α2M resulted in the marked reduction of cell-bound conjugate while the addition of insulin caused only a small decrease in binding. These results indicate that the insulin–α2M conjugate is capable of binding both insulin receptors and α2M receptors and that preferential binding to α2M can be achieved by adding excess insulin.

Next, we examined whether the insulin–α2M conjugate is able to deliver the insulin into cells and if so how it is intracellularly processed. For this, 3T3 cells were incubated with the conjugate for 90 min at 23°C in the presence of excess insulin. Cells were then gently lysed, and the cell-associated radioactivity was analyzed by Percoll density gradient centrifugation (fig.4A). Under these conditions the majority of radioactivity was found in the plasma membrane fraction (ϱ = 1.038) and the rest found in the fractions near the bottom of the gradient. If these cells were further incubated at 37°C, the radioactivity was transferred within 30 min to the bottom fractions. The bottom fractions include lysosomes. In the case of CI-3 cells (fig.4B), the radioactivity was distributed in several fractions including the plasma membrane and lysosomes. The heterogeneous components of intermediate density in CI-3 are likely to be vesicles but at present have not been identified. By raising the temperature to 37°C, the conjugate almost completely shifted to the fractions at the bottom in both cell lines. The radioactivity recovered from the Swiss/3T3 cells which had been treated as in fig.4 are found to be mostly high molecular weight conjugate form (fig.5). Taking all these results together we conclude that the insulin–α2M conjugate is internalized through the α2M receptor and intracellularly processed.

4. DISCUSSION

In this paper, insulin was cystaminylated and cross-linked to the cysteinyl residues of α2M via a disulfide bond. The hybrid molecule had binding capacity for both insulin receptors and α2M receptors. We were able to restrict the binding of the conjugate to either of these receptors by adding insulin or α2M in excess. The binding of the insulin–α2M conjugate to CI-3 cells, which possess a greatly reduced insulin binding capacity, was predominantly mediated through the α2M receptor. We also demonstrated that the conjugate bound to the α2M receptor was internalized and delivered to lysosome-like intracellular organelles.

Recently, Roth et al. [12] produced a conjugate by cross-linking insulin to the binding subunit of ricin (RICb). They demonstrated that the binding of insulin–RICb conjugate to rat hepatoma HTC cells occurred predominantly via the ricin B chain

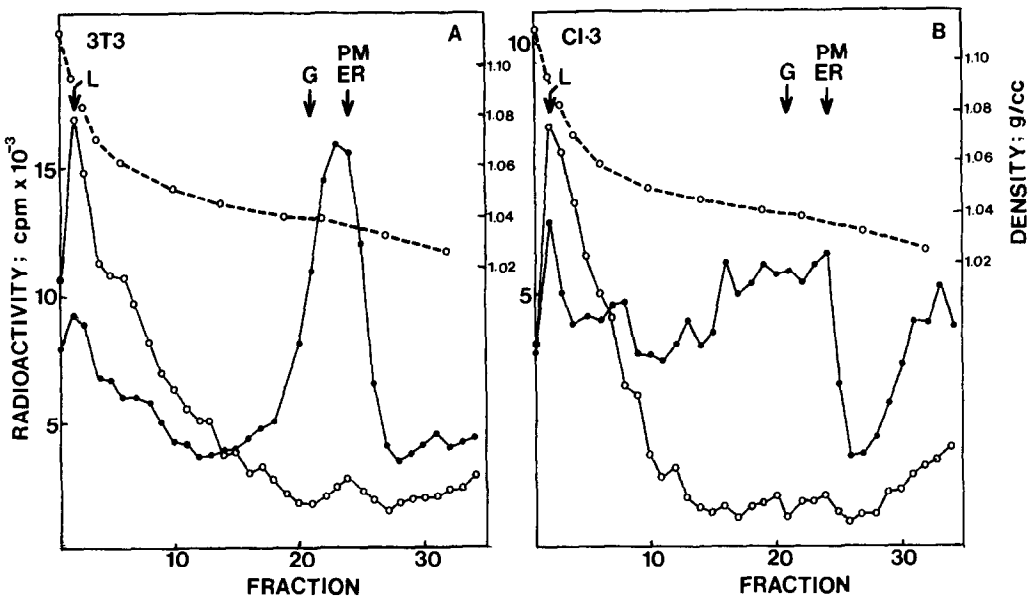

Fig.4. Percoll density gradient analysis of subcellular localization of [^{125}I]insulin–α2M conjugate. Confluent cultures were incubated at 23°C with 0.5 nM [^{125}I]insulin–α2M conjugate in 8 ml of binding buffer containing 1% BSA for 90 min. They were then rinsed and processed for Percoll gradient analysis (—●—). Identically treated cells were further incubated at 37°C in DME-medium containing 10% FCS for 30 min and processed for Percoll analysis (—○—). L, G, PM, and ER denote the position of lysosomes, Golgi, plasma membranes and endoplasmic reticulum, respectively. The position of PM in the gradient was determined by binding of [^{125}I]α2M at 4°C at which temperature α2M binds to the plasma membrane but is not internalized. A density curve (—○—) was determined from refractive index of the gradient fractions.

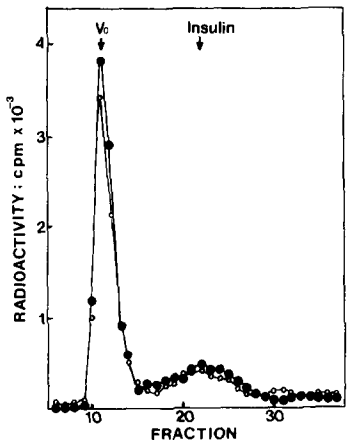

Fig.5. Chromatographic analysis of cell bound [^{125}I]insulin–α2M conjugate. Swiss/3T3 cells were treated with [^{125}I]insulin–α2M conjugate at 23°C for 90 min, washed, and the temperature was shifted to 37°C as in fig.4. At 0 (●) and 30 min (○) after the temperature shift, the cells were dissolved in 4 M urea/1 M acetic acid/0.1% Triton X-100 and analyzed on a column of Sephadex G-75. V_0, void volume.

portion of the conjugate. In the same cells, the conjugate mimicked insulin-like biological activities. However, no data were presented to clarify whether the conjugate was internalized and intracellularly processed to induce such stimulatory effects on the cellular metabolism. The use of RICb for insulin delivery is disadvantageous since insulin and ricin may be transported by separate pathways [13,14]. Utilization of α2M is advantageous because it is known that α2M is bound to rather ubiquitous receptors and internalized in a manner similar to insulin [15]. If our insulin-α2M conjugate is able to produce insulin-stimulated effects in cells, it may be beneficial in the treatment of those diseases which have insulin receptor deficiencies. The utilization of human serum proteins such as α2M will cause no immunological or pathological problems. Moreover, these hybrid insulin molecules would be useful in studying the mechanism of insulin action, particularly to test whether cell surface insulin receptors are essential for activities of insulin.

In preliminary experiments using a denser Percoll gradient, there was clear separation of vesicles containing the conjugate from the lysosomes in both 3T3 and CI-3 cells. Furthermore, large portions of the conjugate remained in the lysosome-like dense vesicles in a high molecular weight form. Interestingly, Khan et al. [16] have recently reported that endocytosed insulin is found in Golgi elements and in unique vesicles having lysosome-like features. The unique vesicles are similar to, if not identical with, the lysosome-like vesicles carrying the internalized α2M–insulin conjugate described here. The biological significance of these vesicles is under investigation, particularly with respect to the possible role of internalized insulin.

ACKNOWLEDGEMENTS

This work was supported by National Institute of Health Grant GM24375 and The Kroc Foundation Grant. We thank K. Miskimins for his contribution to the initial phase of this project and competent technical assistance.

REFERENCES

[1] Kahn, C.R. (1976) J. Cell Biol. 70, 261–286.
[2] Schlessinger, J., Shechter, Y., Willingham, M.C. and Pastan, I. (1978) Proc. Natl. Acad. Sci. USA 75, 2659–2663.
[3] Gorden, P., Carpentier, J.-L., Freychet, P., LeCam, A. and Orci, L. (1978) Science 200, 782–785.
[4] Larner, J., Galasko, G., Cheng, K., DePaoli-Roach, A.A., Huang, L., Daggy, P. and Kellog, J. (1979) Science 206, 1408–1410.
[5] Seals, J.R. and Czech, M.P. (1980) J. Biol. Chem. 255, 6529–6531.
[6] Saltiel, A.R., Siegel, M.I., Jacobs, S. and Cuatrecasas, P. (1982) Proc. Natl. Acad. Sci. USA 79, 3513–3517.
[7] Miskimins, W.K. and Shimizu, N. (1981) Proc. Natl. Acad. Sci. USA 78, 445–449.
[8] Swenson, R.P. and Howard, J.B. (1979) J. Biol. Chem. 254, 4452–4456.
[9] Van Leuven, F., Cassiman, J.J. and Van Den Berghe, H. (1979) J. Biol. Chem. 254, 5155–5160.
[10] Miskimins, W.K. and Shimizu, N. (1979) Biochem. Biophys. Res. Commun. 91, 143–151.
[11] Miskimins, W.K. and Shimizu, N. (1982) J. Cell Physiol. 112, 327–338.
[12] Roth, R.A., Maddux, B.A., Wong, K.Y., Iwamoto, Y. and Goldfine, I.D. (1981) J. Biol. Chem. 256, 5350–5354.
[13] Sandvig, K. and Olsnes, S. (1982) J. Biol. Chem. 257, 7504–7513.
[14] Keen, J.H., Maxfield, F.R., Hardegree, M.C. and Habig, W.H. (1982) Proc. Natl. Acad. Sci. USA 79, 2912–2916.
[15] Maxfield, F.R., Schlessinger, J., Shechter, Y., Pastan, I. and Willingham, M.C. (1978) Cell 14, 805–810.
[16] Khan, M.N., Posner, B.I., Khan, R.J. and Bergeron, J.J.M. (1982) J. Biol. Chem. 257, 5969–5976.