Direct Demonstration of Insulin Receptor Internalization

A Quantitative Electron Microscopic Study of Covalently Bound ^{125}I-Photoreactive Insulin Incubated with Isolated Hepatocytes

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SUMMARY

When ^{125}I-insulin is incubated with isolated rodent hepatocytes at 37°C, the ligand initially binds to the plasma membrane of the cell and is subsequently internalized by adsorptive endocytosis. To confirm directly that the insulin receptor is internalized with the ligand, we covalently linked photoreactive ^{125}I-NΣB29 (azidobenzoyl) insulin to its specific hepatocyte receptor and followed its fate by quantitative electron microscopic autoradiography. We found that the covalently linked photoreactive insulin is internalized by the cell in fashion analogous to the internalization of ordinary ^{125}I-insulin, indicating that, at least under these conditions, the insulin receptor is internalized with the ligand. DIABETES *31*:659–662, July 1982.

It is now clear that, after binding to specific cell surface receptors, most if not all ^{125}I-labeled polypeptide hormones are internalized into a series of intracellular membrane-bound compartments.[1] The first direct morphologic demonstration of this process was made for ^{125}I-insulin incubated with isolated hepatocytes.[2] Polypeptide hormones, like many other ligands that bind to cell surfaces, are internalized by receptor-mediated endocytosis. Since the process is time-, temperature-, and energy-dependent, we postulated that ligand removal from the cell surface is linked to the process whereby cell surface receptors are lost, i.e., homotropic receptor regulation or "downregulation."[1,2] The concordance of ligand internalization and downregulation in systems where the ligand is at least partially irreversibly bound strengthens this argument.[3,4] In addition, in the adipocyte, Marshall and Olefsky have emphasized the close correlation between downregulation of the insulin receptor and events expected to be involved in adsorptive endocytosis.[5] Insulin, for the most part, however, remains at least potentially reversibly bound to its receptor in both hepatocytes and adipocytes.

To demonstrate directly that the insulin receptor can be internalized in isolated hepatocytes, we took advantage of a photoaffinity probe that can be covalently linked to the receptor[6] and followed the fate of covalently linked ^{125}I-insulin by quantitative electron microscopic (EM) autoradiography.

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Received for publication 3 May 1982.

METHODS

Cells and reagents. Rat hepatocytes were isolated by a modification[7] of the method described by Seglen.[8] Before incubation, cell viability, as determined by trypan blue exclusion, was greater than 90%.

The photoreactive insulin, NΣB29 (azidobenzoyl) insulin (MAB insulin), was prepared and iodinated with ^{125}I-(iodine) as previously described.[9,10]

Incubation and photolysis. In Krebs-Ringer bicarbonate (KRB) buffer containing 2% human serum albumin, 0.8 mg/ml bacitracin, and 10 nm ^{125}I-MAB insulin, 1×10^6 cells/ml were incubated in the dark for 30 min at 20°C (referred to as preincubation). Cells were then exposed for 20 s to a filtered (Zeiss BG-3 ultraviolet filter) light source of a high-pressure mercury lamp.[9,10] After photolysis, cells were washed twice with albumin-KRB buffer containing 50 μg/ml unlabeled insulin and once with albumin-KRB alone. After the three wash steps, cells were further incubated at 20°C or 37°C for various time periods when aliquots were removed for the procedures described below, i.e., autoradiography, gamma counting, and gel filtration.

Quantitative electron microscopic autoradiography. At the various times indicated in the results, a 500-μl cell suspension was removed and the radioactivity in the centrifuged pellet determined by gamma counting. The pellet was then fixed, prepared for thin sectioning, coated with emulsion, and further processed for autoradiography exactly as previously described.[2,7,11] Developed autoradiographic grains were photographed on cells that were judged to be

well preserved, and the grain distribution analyzed as previously described.[2,7,11]

Gel filtration and polyacrylamide gel electrophoresis. To determine the cell-associated radioactivity in a high-molecular-weight form (i.e., covalently linked to the receptor) at each time of incubation, an aliquot of cells was washed once in albumin-KRB and then in albumin-KRB containing sucrose. Cells were then centrifuged in a Beckman microfuge (Beckman Instruments Inc., Fullerton, California) and the pellet solubilized in a mixture of triton-urea-acetic acid; the extract was then filtered on a G-50 Sephadex column as described.[7] The void volume fractions, as marked by Dextran blue plus the nonextractable radioactivity (approximately 20–30% of the ^{125}I-MAB insulin is not extractable by the procedures used), represent the high-molecular-weight material. Thus, percent cross-linked ^{125}I-MAB insulin = void volume fraction + nonextractable radioactivity/total cell-associated radioactivity.

Aliquots of the hepatocytes after photolysis were further analyzed by SDS-polyacrylamide gel electrophoresis as described.[9,10] In brief, cells were homogenized in 1 mM sodium bicarbonate, and the crude plasma membrane fraction obtained after centrifugation was solubilized in SDS and reduced with dithiothreitol. The solubilized material was electrophoresed on a 10% SDS-polyacrylamide gel. Autoradiographs were obtained from the dried slab gel.

RESULTS

When ^{125}I-MAB insulin is preincubated with isolated rat hepatocytes in the dark for 30 min at 20°C and then exposed to light to covalently fix a portion of the ligand, a proportion of the radioactivity becomes cell-associated. When cells are fixed, processed, and analyzed by EM autoradiography, the developed autoradiographic grains distribute predominantly around the plasma membrane (~16% of grains are internalized at this time) (Figure 1). When cells are further incubated at 37°C, the grain distribution over a 30-min period shifts toward the inside of the cell, indicating that the cell-associated ^{125}I-MAB insulin is being progressively internalized (Figure 1). By contrast, when the incubation is continued for 30–60 min at 20°C, only a small portion of the ligand is internalized (data not shown).

Since under the conditions of these experiments only a portion of the ^{125}I-MAB insulin is internalized, it is important to relate the covalently linked material to the proportion of the ligand internalized. These data are summarized in Figure 2. After preincubation and photolysis, further incubation of the cells at 37°C leads to a loss of cell-associated radioactivity. This results from a combination of dissociation of noncovalently linked material and some degree of degradation. By 15 min at 37°C, however, the covalently linked fraction is relatively constant, but internalization is progressive between 15 and 30 min at 37°C. We were unable to analyze the 60-min time point at 37°C because of an increase in the number of necrotic cells. By contrast, when cells are incubated at 20°C, more cell-associated radioactivity is retained, i.e., less dissociation and degradation, with only a modest increase in the internalization of the ligand.

To further show that ^{125}I-MAB insulin binds to the subunit structure of the insulin receptor, cell preparations were subjected to SDS-polyacrylamide gel electrophoresis. In the presence of a reducing agent, protein bands of 130k, 90k, and 40k were specifically labeled (Figure 3). This is in agreement with previous studies using isolated liver membranes and isolated rat adipocytes.[9,10] Surprisingly, a 115k band was also reproducibly specifically labeled. This band has not been previously detected and its further identification is presently under study.

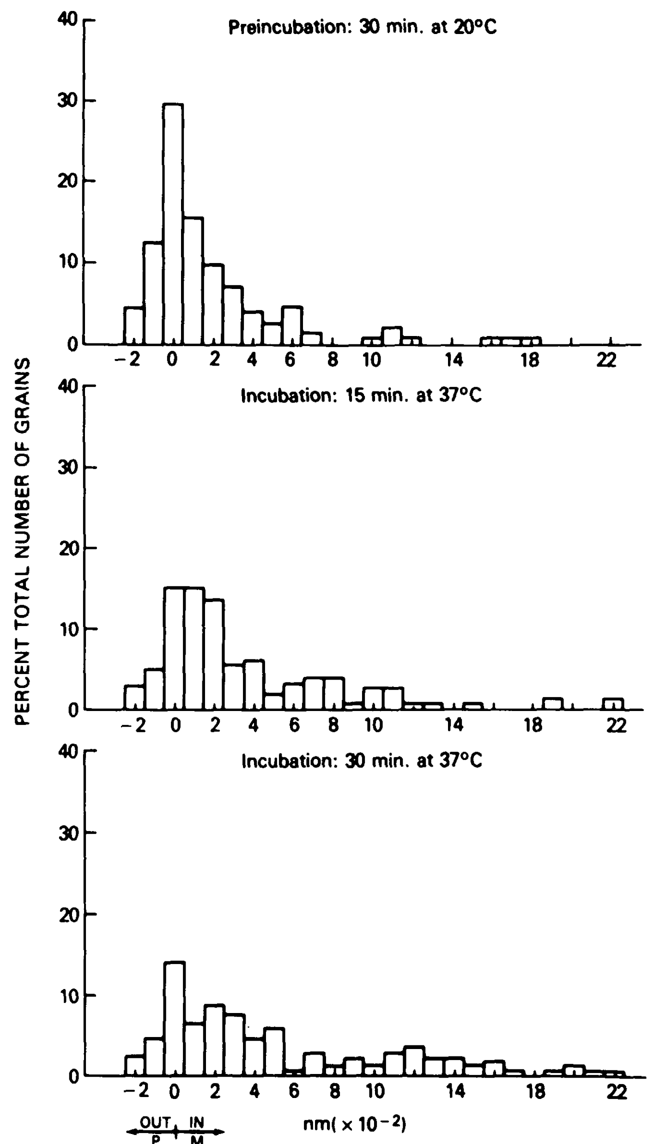

FIGURE 1. Grain distribution histogram: photoreactive insulin incubated with isolated hepatocytes. Approximately 150 individual photographs were made for each developed autoradiographic grain for each time point shown. The percentage of grains (vertical axis) is plotted as a function of the distance from the grain center to the closest plasma membrane (horizontal axis).

DISCUSSION

In the present study we showed that a photoreactive insulin derivative that can be covalently linked to the insulin receptor is internalized by the cell. We interpret this to mean that the insulin receptor can be internalized. These data, cou-

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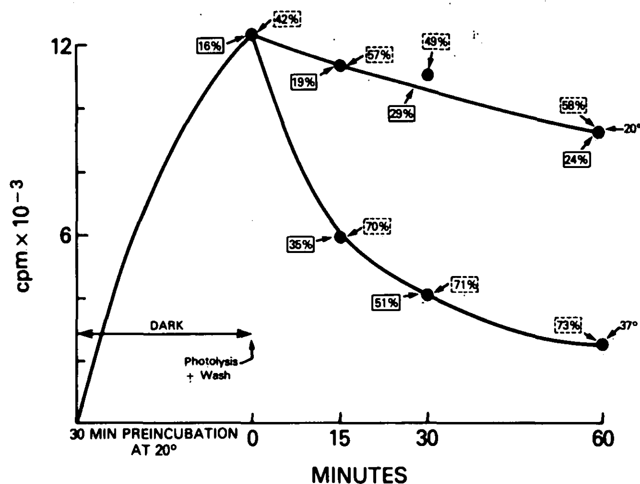

FIGURE 2. Photoreactive insulin interaction with isolated hepatocytes: relationship of cell-associated radioactivity to time and temperature of incubation. Cell-associated radioactivity (vertical axis) is plotted as a function of time (horizontal axis). The percentage figure in the closed box represents the percentage ^{125}I-MAB insulin internalized (for method of calculation see ref. 4). The data for the 37°C experiments are taken from Figure 1 and the data for the 20°C experiments are taken from similarly constructed histograms, which are not shown. The dashed box represents the percentage of cell-associated radioactivity in the high-molecular-weight form (for method of calculation, see METHODS).

pled with an independent study by Fehlmann et al.,[12]* represent the first direct morphologic demonstration of internalization of the insulin receptor. It might be argued, and we cannot directly refute the argument, that the covalent link artificially induces receptor internalization. It is of interest, however, that the overall behavior of the ^{125}I-MAB insulin is remarkably similar to what we have previously observed with ordinary ^{125}I-insulin. At 20°C the internalization rate is slow and at 37°C the rate and extent of internalization is much more rapid.[2,11]

Although photoreactive probes have been used successfully to study the subunit nature of the insulin receptor, this approach is not suitable for the quantitative determination of the amount of receptor because only about 10–20% of the insulin probe is covalently cross-linked to the receptor. In the present study it was essential to establish conditions so that the grains detected by EM autoradiography, on the whole, represent insulin cross-linked to the receptor proteins. This was achieved by extensively washing the cells after photolysis. Thus, at 37°C about 70% of the cell-associated radioactivity remaining after washing was covalently linked to cellular components of high molecular weight that were demonstrated to be subunits of the insulin receptor by electrophoresis and autoradiography.

EM autoradiography makes possible the examination of individual cells to be included in the analysis. In this study, we observed morphologic deterioration of the cell preparation maintained at 37°C for 60 min after photolysis, although apparent cell viability remained at better than 90% as determined by trypan blue exclusion. Consequently, these cells

* The EM autoradiography and analysis in both the present study and the study of Fehlmann et al.[12] were carried out in Geneva. Otherwise, the studies were completely independent. The photoreactive insulin probes were different, the incubation times and temperatures were different, and the experiments were performed in different laboratories.

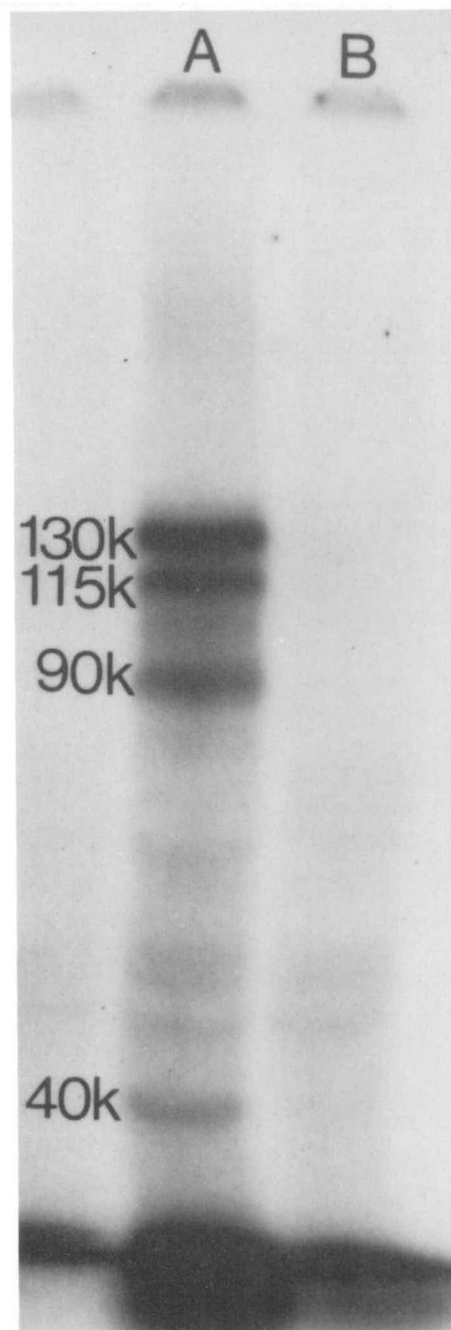

FIGURE 3. Autoradiograph obtained from SDS-polyacrylamide gel electrophoresis of crude plasma membranes of hepatocytes photolabeled with radioiodinated MAB insulin. Lane A shows the specific labeling of receptor subunits of 130kDa, 115kDa, 90kDa, and 40kDa as indicated. Lane B was obtained when excess native insulin was added to the incubation. Previous studies have demonstrated that binding of the photoreactive insulin to liver plasma membranes is inhibited by antibodies to the human insulin receptor and that the anti-insulin receptor antibody will quantitatively precipitate the covalent insulin receptor complex.[13]

were excluded from the analysis. Biochemical methods, such as the use of enzymes or pH changes to affect or alter surface labeling on whole cells, should be applied with caution since the determination of cell viability by dye exclusion can give misleading results.

In summary, we have shown that covalently cross-linked insulin is internalized in the isolated hepatocytes. These

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data further confirm the concept that the receptor is internalized in concert with the ligand and support the idea that ligand internalization is linked to the process of receptor regulation through a process involving adsorptive endocytosis.

ACKNOWLEDGMENTS

This study was supported by Swiss National Science Foundation grant 3.668.80.

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