Transfer of the Hepatocyte Receptor for Serum Asialo-Glycoproteins to the Plasma Membrane of a Fibroblast

ACQUISITION OF THE HEPATOCYTE RECEPTOR FUNCTIONS BY MOUSE L-CELLS*

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Rat liver hepatocytes have oriented externally on their plasma membrane a glycoprotein receptor which can recognize, bind, internalize, and degrade serum glycoproteins from which terminal sialic acid residues have been removed. Mouse L-cells, a tissue culture cell line, like most cells other than hepatocytes, do not have this receptor. A membrane vesicle fraction containing right side out vesicles was prepared from rat liver by sucrose gradient fractionation techniques. These vesicles were enriched 8- to 10-fold over the homogenate in their ability to bind $^{125}$I-asialo-orosomucoid. However, they cannot degrade the iodinated asialo-orosomucoid. The receptor-mediated functions of the mouse L-cell were given to the cells in the culture medium. Although mouse L-cells at 37°C were able to bind, internalize, and degrade $^{125}$I-asialo-orosomucoid which was iodinated in situ using lactoperoxidase-catalyzed iodination as described in detail previously for HTC cells (15), the iodinated membrane vesicles were subjected to two cycles of polyethylene glycol. When L-cells and $^{125}$I-labeled membrane vesicles were subjected to two cycles of polyethylene glycol-mediated fusion, the iodinated membrane vesicles became stably associated with the L-cell membrane. That is, the turnover behavior of the fused liver membrane vesicles was indistinguishable from the plasma membrane of the L-cell. After fusion with liver membrane vesicles, the L-cell at 37°C was able to bind, internalize, and degrade $^{125}$I-asialo-orosomucoid which was given to the cells in the culture medium. Although the iodinated asialo-orosomucoid was degraded to acid-soluble material, the receptors inserted into the L-cell membrane maintained their ability to function through a second cycle of binding, internalization, and degradation of $^{125}$I-asialo-orosomucoid. These experiments show that it is possible to impart to a cell a complex physiological process by inserting into the plasma membrane of that cell the requisite receptor that initiates the sequence of events in the process.

In animal cells, protein and glycoprotein "receptors" that are externally oriented in the plasma membrane are believed to provide the initial steps in complex systems that are responsible for the cell's ability to recognize and respond to changes in the environment (1-3). Externally oriented plasma membrane protein and glycoproteins that can recognize a variety of small and large molecular weight materials external to the cell have been identified in recent years. These include receptors for polypeptide hormones such as insulin (4) and glucagon (5), receptors for lysosomal enzymes (6, 7), receptors for normal serum components such as low density lipoproteins (3, 8), and receptors for altered serum components such as a receptor on liver hepatocytes that can recognize and bind serum glycoproteins that have had their terminal sialic acid residues removed, thus exposing penultimate galactose residues (9-11). The mechanisms by which receptors which are externally oriented on the plasma membrane function to bring about a complex physiological response in the cell is, for the most part, not well understood. Several properties of these receptor proteins have made it difficult to analyze their mechanisms of action. Many of the receptor proteins are present in the membrane at very low concentrations. Further, they are often not confined exclusively to the plasma membrane, but are found in other intracellular membrane systems of the cell (12, 13); the presence of the same receptor in two different membrane compartments complicates enormously mechanistic and biogenetic studies of these membrane receptor proteins (14).

One way to obtain insight into the mechanism of receptor action would be to "induce" a cell to make and insert into its plasma membrane a receptor that it did not previously have. But, it has been very difficult to do this type of experiment rigorously in animal cells. However, in the present study, we use an alternative approach and show that the specific hepatocyte receptor that can recognize serum asialoglycoproteins can be inserted into the plasma membrane of mouse L-cells by cell fusion techniques. The receptor confers upon the mouse L-cell a complex of functions that it did not previously have, i.e. the ability to recognize, bind, internalize, and degrade asialo-orosomucoid. This type of approach offers the possibility of reconstituting and analyzing complex plasma membrane-mediated functions of mammalian cells.

EXPERIMENTAL PROCEDURES

Cells—L-929 cells of mouse origin were obtained from Dr. Eric Mayhew, Roswell Park Memorial Institute. They were grown and maintained as monolayer cultures in RPMI 1640 medium containing heat-inactivated fetal calf serum (10%) at 37°C in a 5% CO$_2$ incubator. L-cells were iodinated in situ using lactoperoxidase-catalyzed iodination as described in detail previously for HTC cells (15). Preparation of a Plasma Membrane Fraction from Rat Liver—Plasma membrane fractions P$_1$ and N$_2$ were prepared from Sprague-Dawley rats, 250 to 500 g, exactly as described by Aronson and Touster (16). The sucrose used in the fractionation was from Serva (Accurate Chemical and Scientific Corp., Hicksville, N. Y.); sucrose from Serva, in contrast to that from other suppliers, did not inhibit the binding of asialo-orosomucoid by the membrane fraction. The P$_1$ fraction which was higher in asialoglycoprotein binding than the N$_2$ fraction of the liver plasma membrane was iodinated using the chloramine-T procedure (17). Typically, 2.4 mg of P$_1$ plasma membrane protein was reacted with 1 to 2 mCi of $^{125}$I (Amersham) in the presence of 50 mM Hepes buffer, 50 mM NaCl, pH 8.0, and 2.6 mg/ml of chloramine-T. The volume of the reaction mixture was kept as small as possible, usually less than 0.1 ml. The reaction was stopped after less than 1 min by the addition of sodium metabisulfite (0.28 mg/ml) and 1 ml of Hepes buffer containing 0.05 M NaCl. The membranes were collected by ultracentrifugation at 50,000 rpm for 30 min and were washed once with the Hepes/sodium chloride buffer.

Fusion of Rat Liver P$_1$ Plasma Membrane Vesicles with Mouse...
L-Cells (18)—Monolayer cultures of confluent L-929 fibroblasts in 75-cm² flasks (Costar, Cambridge, Mass.) were treated with trypsin from Gibco (0.5 mg/ml) for 5 min at 37°C. The suspended cells were washed twice with phosphate-buffered saline. To the packed cell pellet containing as little phosphate-buffered saline as possible was added newly prepared P₂ plasma membrane fraction of liver cells, 1 to 2 mg of liver membranes in about 0.1 ml/1 × 10⁷ cells/fusion. The components were mixed well by stirring with a Pasteur pipette. One milliliter of 50% (w/v) polyethylene glycol (M₇ = 1000, J. T. Baker Chemical Co.) in serum-free medium was added for 1 min at room temperature. Then, 15 ml of serum-free medium was added. After 2 min more, the cells were collected by centrifugation and were washed once with serum free medium. A second identical cycle of fusion was performed after which the cells were washed twice with serum-free medium, and placed back in monolayer culture at 37°C. The cells were 90 to 90% viable after the second cycle of fusion.

Binding Assay for Asialo-Orosomucoid—Orosomucoid was a generous gift of Dr. Milan Wickerhauser of the Blood Research Laboratory of the American National Red Cross. Terminal sialic acids were removed with neuraminidase (insoluble neuraminidase coupled to agarose was from Sigma). Orosomucoid, 4 mg, was incubated with 0.023 units of neuraminidase in 0.15 M acetate buffer, pH 5.6, at 37°C for 6 to 19 h. The release of sialic acid was monitored using the thiobarbituric acid assay of Warren (19). Asialo-orosomucoid and orosomucoid were iodinated with ¹²⁵I using lactoperoxidase coupled to Sepharose 4B (Pharmacia Fine Chemicals) according to the method of David and Reisfeld (20), or by the chloramine-T procedure (17). The capacity of the iodinated asialo-orosomucoid to bind specifically to its receptor varied somewhat depending on method of preparation. Specific activities of the iodinated proteins and method of preparation are given in the legends to the figures.

Assay A of Hudgin et al. (21) was used to assay the binding of iodinated asialo-orosomucoid by isolated membrane fractions.

Counting Procedures—¹²⁵I-labeled material was counted directly in a Beckman Biogamma spectrometer. Acid-insoluble and acid-soluble radioactivity in cells or in the medium from cells was determined by first treating with 1% phosphotungstic acid (in 0.5 N HCl) or 10% trichloroacetic acid (15). Phosphotungstic acid was used to precipitate samples containing labeled asialo-orosomucoid or orosomucoid. The phosphotungstic acid mixtures were placed on ice for 20 min and then centrifuged at 10,000 rpm for 10 min. Acid-soluble material was removed and counted. The pellet was washed twice with ethanol by sonication and centrifugation. The pellet was dissolved in 1 ml of 1 N NaOH and counted.

RESULTS AND DISCUSSION

A plasma membrane fraction P₂ can be isolated from rat liver by the method of Aronson and Touster (16) which is identical. That is, none of these cell lines can distinguish between L-Cells, Monolayer cultures of confluent L-929 fibroblasts in 75-cm² flasks (Costar, Cambridge, Mass.) were treated with trypsin from Gibco (0.5 mg/ml) for 5 min at 37°C. The suspended cells were washed twice with phosphate-buffered saline. To the packed cell pellet containing as little phosphate-buffered saline as possible was added newly prepared P₂ plasma membrane fraction of liver cells, 1 to 2 mg of liver membranes in about 0.1 ml/1 × 10⁷ cells/fusion. The components were mixed well by stirring with a Pasteur pipette. One milliliter of 50% (w/v) polyethylene glycol (M₇ = 1000, J. T. Baker Chemical Co.) in serum-free medium was added for 1 min at room temperature. Then, 15 ml of serum-free medium was added. After 2 min more, the cells were collected by centrifugation and were washed once with serum free medium. A second identical cycle of fusion was performed after which the cells were washed twice with serum-free medium, and placed back in monolayer culture at 37°C. The cells were 90 to 90% viable after the second cycle of fusion.

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RESULTS AND DISCUSSION

A plasma membrane fraction P₂ can be isolated from rat liver by the method of Aronson and Touster (16) which is about 8- to 10-fold enriched over the homogenate in its ability to bind ¹²⁵I-asialo-orosomucoid (Table I). This liver cell plasma membrane fraction in the electron microscope appears as a heterogeneous population of closed membrane vesicles with an average diameter of about 1 μm (22). The fact that these closed vesicles can bind asialo-orosomucoid, a glycoprotein of M₇ = ~40,000, indicates that at least some of them are oriented in a right side out configuration.

The plasma membrane receptor that can recognize and bind the penultimate galactose residues of asialoglycoproteins is rather specifically confined to hepatocytes. No tissue culture cell line that we have examined, including hepatoma tissue culture cells lines which are of rat hepatocyte origin (HTC cells) or mouse hepatocyte origin (Hepa) or a mouse fibroblastic cell line (L-929 cells) binds asialoglycoproteins specifically. That is, none of these cell lines can distinguish between ¹²⁵I-orosomucoid and ¹³¹I-orosomucoid from which the terminal sialic acids have been removed either chemically or by neuraminidase.

In contrast, the membrane vesicles derived from rat liver are very efficient in their ability to bind asialo-orosomucoid; 1 mg of these vesicles will bind about 600 ng of asialo-orosomucoid at saturation (Fig. 1). These vesicles show no high affinity specific binding of orosomucoid nor are they capable of degrading the bound asialo-orosomucoid to acid-soluble material. Hence, the vesicles represent, most likely, right side out closed plasma membrane fragments derived from the sinusoidal face of the rat hepatocyte (16, 22).

The rat liver membrane vesicles can be “fused” to tissue culture cells in the presence of polyethylene glycol. When liver membrane vesicles are first iodinated via lactoperoxidase-catalyzed iodination and then fused with L-cells, the radioactive iodide becomes stably associated with the L-cell (Fig. 2). Indeed, the radioactive iodide of the liver membrane vesicle is as stable as the iodide incorporated into the membrane proteins of the L-cell itself (Fig. 2). The iodinated liver membrane proteins inserted into L-cells or HTC cells by polyethylene glycol-mediated fusion have similar and almost identical turnover characteristics as the iodinated proteins of the plasma membrane of the recipient cell (22). The experiment presented in Fig. 2 shows that the iodinated membrane proteins of the L-cell as well as those iodinated liver membrane proteins inserted into the L-cell by polyethylene glycol-mediated fusion turn over with relatively long half-lives. Further, the presence of asialo-orosomucoid in the medium has no effect on the turnover of the iodinated hepatocyte proteins introduced into the L-cell. Elsewhere, we will show that the hepatocyte receptor for asialoglycoproteins, as assayed with a specific antibody, also has a half-life similar and probably identical with the other iodinated proteins of the liver mem-

3 E. Hou, R. Warren, and D. Doyle, manuscript in preparation.
4 R. Warren, and D. Doyle, manuscript in preparation.
with trypsin and then reacted with polyethylene glycol through two cycles of the chloramine-T procedure (specific activity, 6.7 protein). Confluent L-929 cells from one 75-cm² flask were removed of rat liver plasma membrane was iodinated with °S'I via the chloramine-T procedure and then distributed among twenty plates (35 X 10 mm). A confluent culture of L-cells were washed twice with serum-free medium and then distributed among twenty plates (35 X 10 mm) (G, lower graph). Eight hours later, asialo-orosomucoid (5 μg/ml) was added to some plates (upper graph). At the times indicated, asialo-orosomucoid was added back to these cells. Trichloroacetic acid-insoluble radioactivity was assessed at the times indicated. Zero time is immediately after the fused cells were placed on the plates (35 X 10 mm). A confluent culture of L-cells growing as a monolayer in one 75-cm² flask was iodinated via lactoperoxidase-catalyzed iodination, 0.5 mCi of °S'I/flask. The cells were then removed from the flask with trypsin and were distributed among ten plates (35 X 10 mm) (Q, lower graph). Trichloroacetic acid (10%)-insoluble radioactivity was determined as described previously (14).

brane and the host cell membrane after fusion of liver membranes. Long half-lives or slow turnover times relative to the doubling time of the cell seems to be a characteristic property of plasma membrane proteins of many different types of cells in culture (1, 23-25).

To demonstrate that the biological response that is specified by the hepatocyte receptor is transferred to the recipient cell intact, we assayed for function by measuring the uptake and degradation of asialo-orosomucoid. As shown in Fig. 3, L-cells containing the fused liver membrane, in contrast to the parent L-cell, can bind, take up, and degrade °S'I-asialo-orosomucoid. Two cycles of fusion were used to transfer the liver membranes to the L-cell because, under optimal conditions, only about 1 to 5% of the liver membranes will become associated with the recipient cell. Two cycles of fusion allow a sufficient number of receptor proteins to be inserted into the L-cell to permit assay of function. It should be mentioned that the °S'I fraction of rat liver, while heavily enriched in plasma membrane fragments, is not homogeneous. Since the receptor for asialo-orosomucoid is also present in other membrane fractions of rat liver (12), there is some possibility that membranes other than the plasma membrane transfer the receptor to the L-cell. In Fig. 3, the cells after fusion were placed back in culture in the presence of °S'I-asialo-orosomucoid (5 μg/ml). This concentration of asialo-orosomucoid is very much in excess of the amount that can be processed in the L-cell by the relatively limited number of receptors that have been inserted even by two cycles of fusion. For example, at most, 100 μg of liver membranes is transferred to 10⁵ cells after two cycles of fusion in which 1 mg of liver membrane is reacted twice with 10⁵ cells. As shown in Fig. 1, this amount of liver membrane can only bind on the order of 60 ng of the asialo-orosomucoid at saturation at 4°C. Elsewhere (14), we will show that after 6 to 8 h, the amount of asialo-orosomucoid associated with the L-cell reaches a plateau. The time required to reach this plateau represents the time required to achieve a steady state of binding, interiorization, and degradation of the asialo-orosomucoid by the cells relative to the concentration of the asialo-orosomucoid in the medium. This time of about 6 to 8 h for the modified L-cells is actually very similar to the time required by primary cultures of isolated rat hepatocytes to do the same series of reactions and attain a steady state between the cell-associated asialo-orosomucoid and that in the

\[ \text{Time (hours)} \]

\[ 0 \quad 10 \quad 20 \quad 30 \quad 40 \quad 50 \quad 60 \]

\[ \text{125I Acid-Insoluble Radioactivity} \]

\[ \text{cpm/mL} \]

\[ 0 \quad 10 \quad 20 \quad 30 \quad 40 \quad 50 \quad 60 \]

\[ \text{Laniece fused with 125I-Labeled Liver Membranes} \]

\[ \text{A} \]

\[ \text{B} \]

\[ \text{C} \]

\[ \text{D} \]

\[ \text{E} \]

\[ \text{F} \]

\[ \text{G} \]

\[ \text{H} \]

\[ \text{I} \]

\[ \text{J} \]

\[ \text{K} \]

\[ \text{L} \]

\[ \text{M} \]

\[ \text{N} \]

\[ \text{O} \]

\[ \text{P} \]

\[ \text{Q} \]

\[ \text{R} \]

\[ \text{S} \]

\[ \text{T} \]

\[ \text{U} \]

\[ \text{V} \]

\[ \text{W} \]

\[ \text{X} \]

\[ \text{Y} \]

\[ \text{Z} \]

\[ \text{insertion and function of membrane receptors in foreign cells} \]

\[ 6855 \]

\[ \text{FIG. 2. Turnover of °S'I-labeled plasma membrane proteins of L-cells and °S'I-labeled liver membrane proteins inserted into L-cells by fusion with polyethylene glycol. The °S'I fraction of rat liver plasma membrane was iodinated with °S'I via the chloramine-T procedure (specific activity, 6.7 x 10³ cpm/mg of membrane protein). Confluent L-929 cells from one 75-cm² flask were removed with trypsin and then reacted with polyethylene glycol through two cycles of fusion with the liver membranes. (A, lower graph). Eight hours later, asialo-orosomucoid (5 μg/ml) was added to some plates (upper graph). At the times indicated, asialo-orosomucoid was added back to these cells. Trichloroacetic acid-insoluble radioactivity was assessed at the times indicated. Zero time is immediately after the fused cells were placed on the plates (35 X 10 mm). A confluent culture of L-cells growing as a monolayer in one 75-cm² flask was iodinated via lactoperoxidase-catalyzed iodination, 0.5 mCi of °S'I/flask. The cells were then removed from the flask with trypsin and were distributed among ten plates (35 X 10 mm) (Q, lower graph). Trichloroacetic acid (10%)-insoluble radioactivity was determined as described previously (14).} \]

\[ \text{FIG. 3. Metabolism of asialo-orosomucoid by L-cells fused with rat liver plasma membranes. L-929 cells from two confluent 75-cm² flasks (about 10⁵ cell/flask) were fused with the °S'I fraction of rat liver as described under "Experimental Procedures" and in the legend to Fig. 2. The cells were distributed among 40 plates (35 X 10 mm) which were incubated at 37°C overnight. The next day, the attached cells were washed three times with phosphate-buffered saline. Medium (1 ml), containing °S'I-asialo-orosomucoid (4 x 10⁶ cpm, 10 ng, iodinated via the lactoperoxidase-Sepharose method) and 5 μg of unlabeled asialo-orosomucoid was added to each culture. Cell-associated phosphotungstic acid-insoluble radioactivity was determined at the times indicated. Cells were washed three times with phosphate-buffered saline and material insoluble in phosphotungstic acid was counted in the Biogamma spectrometer. After 8 h in the presence of asialo-orosomucoid, the medium was removed from the cells and the cells were washed twice with medium not containing asialo-orosomucoid. Phosphotungstic acid-soluble radioactivity in the medium and acid-insoluble radioactivity associated with the cells was determined at the times indicated. At the time indicated, °S'I-asialo-orosomucoid (4 x 10⁶ cpm) and unlabeled asialo-orosomucoid, 5 μg, were added back to the cell cultures. Acid-insoluble radioactivity associated with the cells was assayed. Finally, the asialo-orosomucoid in the medium was removed by washing the cells again and cell-associated acid-insoluble radioactivity and acid-soluble radioactivity in the medium were determined. The L-cell before fusion with the liver membranes will bind 500 cpm or less of asialo-orosomucoid when added at the same concentration and under the same conditions as specified above for the fused cells.} \]
...Hence, we presume that the L-cell is functioning very much like the hepatocyte in this series of reactions. The individual steps in the series can be analyzed separately and we will report on the properties of the binding, internalization, and degradation steps in more detail in subsequent communications. We point out here that binding of the asialoglycoprotein by the receptor is a necessary but not the only prerequisite for the cell to accomplish internalization and degradation. The L-cell is supplying some components for these latter steps because we can show that HTCC cells after polyethylene glycol-mediated fusion with liver membranes can bind asialo-lysosomal glycoproteins, but the modified HTCC cells cannot degrade the bound protein. When the asialo-orosomucoid is removed from the medium of the modified L-cell at about the time of the plateau, between 5 and 10 h in Fig. 3, the cell will complete the degradation of the asialo-orosomucoid still associated with the cell to small molecular weight acid-soluble material (Fig. 3).

The hepatic asialoglycoprotein receptor once inserted into the L-cell is, as mentioned above, stable and apparently is not degraded during the steps involved in the degradation of the bound asialoglycoprotein. These steps, as mentioned, presumably include binding, internalization of membrane units containing the receptor and bound asialoglycoprotein, fusion with lysosomes, and degradation of the asialoglycoprotein to acid-soluble material (1, 23, 26). If these steps, indeed, are involved in the mode of this receptor action, they do not affect significantly the receptor itself because the modified L-cell, after internalizing and degrading the bound asialo-orosomucoid, is still capable of binding and degrading asialo-orosomucoid when the cells are exposed for a second time to high concentrations of the glycoprotein in the medium (Fig. 3). Hence, the hepatic receptors are still present and functional on the cell surface. Also, it is possible to demonstrate by fluorescence microscopy that hepatocyte membranes are localized at the surface of L-cells after fusion with liver vesicles. An explanation for the ability of the hepatic receptor for asialoglycoproteins to function for extended times after its insertion into L-cells while the asialoglycoprotein that it binds is degraded is that the receptor is capable of cycling in and out of the cell surface. Tanabe et al. (27) recently have presented some evidence for this type of receptor recycling in intact rats and we also have evidence that recycling is part of the reason for the stability of the asialoglycoprotein receptor in isolated rat hepatocytes. Fig. 3 also shows that there is a slow component in the degradation of the asialo-orosomucoid. This is seen most easily after the asialo-orosomucoid is removed from the medium between 25 and 30 h. Isolated hepatocytes also show this type of behavior (28), the biphasic kinetics are due to high affinity receptor-mediated turnover and also a low affinity nonreceptor-mediated turnover of the asialoglycoprotein.

The remarkable biological stability of plasma membrane proteins coupled with the ability to insert foreign membrane proteins into a tissue culture cell of choice and have these proteins function should help to resolve some of the steps involved in the mechanism of receptor action. It should be mentioned that it is possible that this method can be used to put any foreign membrane protein into a recipient cell. Indeed, while our experiments were in progress, a report appeared showing that similar techniques could be used to activate a cell's adenyl cyclase by fusion of a foreign membrane-bound glucagon receptor (29). Finally, since several cycles of polyethylene glycol-mediated fusion can be done on one cell, it might be possible to supply more than one component of a complex system to the recipient cell. In this way, it might be possible to "reconstitute" in the recipient cell a complex membrane-mediated response by obtaining the component pieces from other cells lacking one or more of the membrane proteins involved in the response.

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