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Research Article

Comparison of the Methods for Determining Cell-Surface and Intracellular Receptors for Epidermal Growth Factor in the Rat Liver

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We compared methods for determining the distribution of epidermal growth factor (EGF) receptors between the cell surface and the cell interior in the rat liver. Incubation of isolated hepatocytes with 100 n*M* EGF for 20 min at 37°C remarkably decreased the cell-surface EGF receptor density (internalization of receptors). The detergent Brij 35 was previously reported to permit assay of the intracellular latent EGF receptors in liver homogenates, but in the present investigation, Brij 35 lowered the affinity of EGF for the receptor depending on the detergent concentration, and the appearance of latent receptors was not observed. In contrast, permeabilization of the cells with digitonin, followed by an acid-washing procedure, increased the EGF binding capacity to close to the control level. Hence, the EGF receptors, internalized together with EGF molecules, were not degraded for at least 20 min, and the digitonin method is suitable for quantifying the intracellular EGF receptors. The binding capacities of the digitonin-treated and untreated control cells showed no difference upon digitonin treatment, suggesting that the bulk of EGF receptors exists on the cell surface. Further, cell-surface EGF receptor density was determined after the i.v. administration of EGF (300 μg/kg) to rats. Isolated hepatocytes prepared 30 min after the administration of EGF showed little binding for EGF on the cell surface, while the cell-surface EGF receptor density recovered to close to control values in cells prepared after 3 hr.

KEY WORDS: epidermal growth factor receptor; rat liver; digitonin; Brij 35; intracellular receptor; latent receptor; down-regulation; acid washing.

INTRODUCTION

Measuring the density of the cell-surface and intracellular receptor for various neuro- and hormonal polypeptides is necessary to study receptor dynamics. The receptors for certain polypeptides, such as low-density lipoprotein, asialoglycoprotein, and transferrin, recycle to the cell surface from the intracellular compartment following receptor-mediated endocytosis. On the other hand, some receptors such as the interferon receptor are degraded after being internalized. Kinetic studies of the uptake of epidermal growth factor (EGF) by various cells were performed using *in vitro* (1–4) and *in vivo* (5–7) experimental systems. Dunn *et al.* (8) demonstrated the degradation of EGF receptors in the perfused rat liver with a half-life of 2.5 hr, but receptor recycling was also demonstrated in the same system (8,9). Suitable methods are needed to quantitate recycling or degradation of the receptors internalized with EGF. We have tested here whether assays developed for internalized insulin and asialoglycoprotein receptors employing trypsin digestion (10) or digitonin permeabilization (11,12) are also applicable to the intracellular EGF receptors of rat hepatocytes. Dunn and Hubbard (9) reported that the latent (intracellular) EGF receptors can be detected after treatment of the liver homogenates with the nonionic detergent Brij 35, and this method was also tested for comparison.

We have previously reported the kinetics of EGF receptor down-regulation in rat liver after i.v. administration of excess EGF (300 μg/kg) *in vivo* (6). A remarkable decrease in the early-phase hepatic uptake clearance of ^{125}I-EGF which is proportional to the available cell-surface receptors was observed 30 min after EGF administration, and thereafter, the clearance gradually recovered to control levels. We also observed a similar recovery of the available cell-surface receptors in the rat liver perfusion systems (13). In the present study, we therefore tested whether the cell-surface EGF receptor density indeed changes in parallel with the EGF clearance data obtained *in vivo* and in the liver perfusion systems.

MATERIALS AND METHODS

Materials. Epidermal growth factor (EGF) was supplied by Wakunaga Pharmaceutical Co., Ltd. (Hiroshima, Japan), sodium iodide-125 (100 mCi/ml) was purchased from the Radiochemical Center (Amersham Co., Arlington

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Heights, IL), bovine serum albumin (BSA; fraction V) from Sigma Chemical Co., (St. Louis, MO), Sephadex G-25 from Pharmacia Fine Chemicals (Uppsala, Sweden), and collagenase, trichloroacetic acid (TCA), *n*-butyl phthalate, and Brij 35 from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were commercial products of analytical grade.

Synthesis of ^{125}I-EGF. Biosynthetic EGF obtained from *Escherichia coli* via the synthesized coding sequence described previously (14) was used in all experiments. EGF was radiolabeled with ^{125}I-Na by the chloramine-T method (15). Unreacted ^{125}I-Na was removed by a Sephadex G-25 column, and the ^{125}I-EGF was eluted in the void volume. The ^{125}I-EGF had a specific activity of 0.5–1.0 mCi/nmol and was >95% precipitable in 15% trichloroacetic acid (TCA). Further, >98% of ^{125}I-EGF binding to a specific antiserum was displaced by excess unlabeled EGF (6 n*M*).

Preparation of Hepatocytes. Male Wistar rats (200–230 g) housed under conditions of controlled temperature and lighting with access to food and water ad libitum were used throughout the experiment. Hepatocytes were prepared by the procedure of Baur *et al.* (16) with a modification. Briefly, under urethane anesthesia (1 g/kg) the liver was perfused with about 200 ml of a calcium-free bicarbonate buffer. The liver was subsequently perfused for 15 min with the collagenase (0.05%) medium containing calcium (1 m*M*). The liver was then gently swayed in the calcium-free bicarbonate buffer and the parenchymal cells were purified by several filtration and centrifugation procedures. After washing, hepatocytes were resuspended in standard buffer containing 0.1% BSA, 130 m*M* NaCl, 5 m*M* KCl, 0.5 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 20 m*M* Hepes, and NaOH to give a pH of 7.4.

EGF Binding Assay. The cells or cell homogenates were incubated with the tracer amount of ^{125}I-EGF (0.1 μCi/ml) and increasing concentrations (0.5–100 n*M*) of unlabeled EGF for 90 min at 0°C. After incubation, the amounts of EGF bound to the cells and cell homogenates were measured by the centrifugal filtration method and rapid filtration method, respectively, as described previously (17).

Acid-Washing Method. An acid-washing method was used to dissociate EGF bound by the cell-surface receptors using a pH 5.2 buffer (18). The acid-washed cells maintain EGF binding comparable with the normal cells. The pH 5.2 buffer contained 0.1% BSA, 130 m*M* NaCl, 5 m*M* KCl, 0.5 m*M* $MgCl_2$, 20 m*M* Mes, and NaOH to give a pH of 5.2. Cells were exposed to the mild acid buffer on the ice for 2 min, then washed twice with the ice-cold standard buffer (pH 7.2). For comparison, a commonly used pH 3.1 buffer (0.2 *M* acetic acid and 0.5 *M* NaCl, pH 3.1) (19) was also used to assess surface-bound EGF molecules. The cell-associated radioactivity which was resistant to acid treatment is taken to represent internalized EGF.

Comparison of EGF Binding by Hepatocytes and Their Homogenates Isolated from Control and EGF-Treated Rats. Hepatocytes were prepared from control rats and from rats 30 min or 3 hr after i.v. administration of unlabeled EGF (300 μg/kg). Aliquots of these cell suspensions were homogenized with a Teflon homogenizer to allow exogenous EGF molecules to gain access to intracellular EGF receptors. Then the binding assays were performed using the cells and the cell homogenates, respectively.

Comparison of EGF Binding of Untreated and Digitonin-Treated Hepatocytes. Hepatocyte suspensions (obtained from rats that were not treated with EGF) were incubated for 20 min at 37°C with 100 n*M* unlabeled EGF to obtain cells with mostly internalized EGF receptors. Digitonin fractionation was performed by the method of Andersson *et al.* (20) with a slight modification. Normal cells and cells with mostly internalized receptors (cellular protein concentration, 1.6 mg/ml) were incubated for 2 min at room temperature with 0.24 mg/ml digitonin in 3.3 m*M* MES buffer containing 3.3 m*M* EDTA (disodium salt), 125 m*M* NaCl, pH 7.4. After pH 5.2 acid washing, EGF binding was determined.

Comparison of EGF Binding by Liver Homogenates from Control and EGF-Treated Rats Determined Using Brij 35. EGF binding to the liver homogenates, prepared from control rats and rats sacrificed 30 min after i.v. administration of unlabeled EGF (300 μg/kg), was measured according to the method of Dunn and Hubbard (9). Samples were incubated with ^{125}I-EGF (0.1 μCi/ml) and unlabeled EGF (0.5–80 n*M*) in the presence and absence of Brij 35 (1 g/dl) on ice for 90 min in a total volume of 0.2 ml that contained 20 m*M* Hepes, (pH 7.4) and 0.5% BSA. The EGF-receptor complex was precipitated with polyethylene glycol and collected on Whatman GF/C filters.

Trypsin Digestion Method. The control cells and the cells with mostly internalized EGF receptor were washed with the pH 5.2 buffer. Aliquots of these cells were incubated in the standard buffer containing 0.1% trypsin for 30 min at 0°C, then washed twice with the ice-cold standard buffer containing 0.005% trypsin inhibitor. Aliquots of the trypsin-treated cells were homogenized with a Teflon homogenizer to assess the quantity of intracellular receptors. The binding assays were then performed on the cells, trypsin-treated cells, and trypsin-treated cell homogenates, respectively.

Data Analysis. The binding data were analyzed on the assumption of a single class of binding sites and nonspecific binding as follows:

$$r = C_b/P_T = (n \cdot C_f)/(K_d + C_f) + \alpha \cdot C_f \quad (1)$$

where C_b and C_f are the bound and unbound concentrations of EGF, respectively, r is the amount of bound EGF per milligram of cellular protein, P_T is the cellular protein concentration, n and K_d denote the specific binding capacity per milligram of cellular protein (receptor density) and the dissociation constant, respectively, and α is the proportional constant related to the nonspecific binding. The binding parameters (n, K_d, and α) were obtained by an iterative nonlinear least-squares method (21). The initial estimates of the parameters were obtained from Scatchard plots.

Simulation Study. In the binding assay using liver homogenates prepared from EGF-treated rats, EGF molecules originating from the preadministered EGF may make it difficult to estimate the receptor densities *in vitro*. To understand the effect of preadministered EGF on the experimentally obtained Scatchard plots, a simulation study was performed. The apparent amount of bound EGF (r) is given as

$$r = (n \cdot C_f)/(K_d + C_{pre} + C_f) + \alpha \cdot C_f \quad (2)$$

where C_{pre} is the unbound concentration coming from preadministered EGF. This equation holds if we assume a complete redistribution between preadministered EGF and that added *in vitro* and if the unbound concentrations of EGF (C_f) are assumed to be equal to the total concentrations.

RESULTS

Comparison of EGF Binding Isotherms Between the Livers from Control and EGF-Treated Rats

Figure 1 shows the Scatchard plots for EGF binding to isolated hepatocytes and their homogenates prepared from control rats and from rats sacrificed 30 min (named "DR0.5 rat") or 3 hr ("DR3 rat") after the i.v. administration of 300 μg/kg unlabeled EGF. These data were fitted to Eq. (1), and the parameter values obtained are summarized in Table I. In control rats, the binding parameters were comparable between the cells and the homogenates. The cell-surface receptor density assessed from the binding capacity (n) in DR0.5 cells greatly decreased, while the cell surface receptor density recovered to control in the DR3 cells. However, the K_d value for DR3 cells is approximately three times that

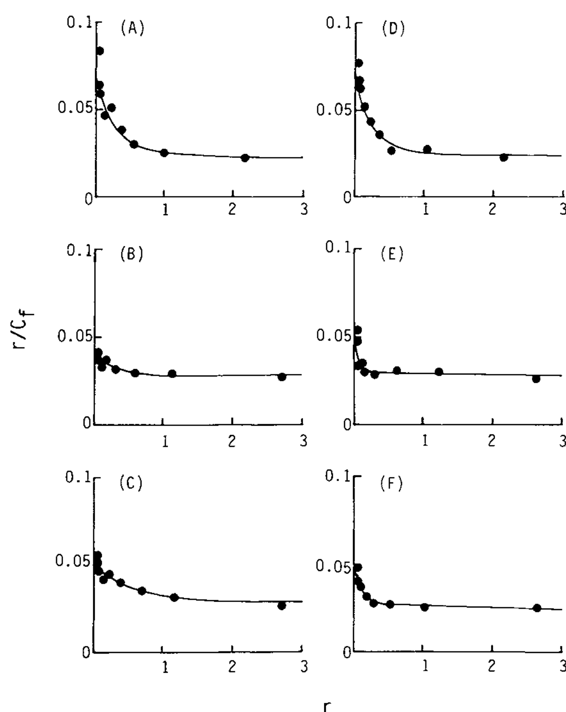

Fig. 1. Scatchard plots of EGF binding to isolated rat hepatocytes and their homogenates prepared from control rats and rats sacrificed 30 min or 3 hr after i.v. administration of 300 μg/kg unlabeled EGF. (A) Cells from control rats, (B) cells from rats 30 min after EGF administration, (C) cells from rats 3 hr after EGF administration, (D) cell homogenates from control rats, (E) cell homogenates from rats 30 min after EGF administration, and (F) cell homogenates from rats 3 hr after EGF administration. r is the amount of bound EGF per milligram of cellular protein, and C_f is the unbound concentrations of EGF. The data points are the means of three or four independent experiments, and the standard deviations of all the data points are within 15% of the mean values. Solid lines were calculated by an iterative nonlinear least-squares method based on Eq. (1).

Table I. Parameter Values for EGF Binding to Isolated Rat Hepatocytes and Their Homogenates Prepared from Control Rats and Those 30 min (DR 0.5) or 3 hr (DR 3) After the i.v. Administration of 300 μg/kg Unlabeled EGF[a]

	n (pmol/mg protein)	K_d (nM)	α (ml/mg protein)
Control cell	0.26 ± 0.09	4.9 ± 1.8	0.019 ± 0.002
Control-cell homogenates	0.19 ± 0.03	3.5 ± 0.5	0.020 ± 0.001
DR 0.5 cells	0.067 ± 0.039	4.8 ± 2.8	0.027 ± 0.001
DR 0.5-cell homogenates	0.011 ± 0.004	0.21 ± 0.19	0.029 ± 0.002
DR 3 cells	0.33 ± 0.14	13 ± 5	0.025 ± 0.002
DR 3-cell homogenates	0.048 ± 0.014	1.7 ± 0.6	0.026 ± 0.001

[a] The data shown in Fig. 1 were fitted to Eq. (1) by an iterative nonlinear least-squares method. The values are the means ± SE of three or four independent determinations.

of control cells. The reason for such increase in K_d value for DR3 cells is not known yet, but EGF molecules could have been released from the liver into the medium during the cell isolations, thereby increasing the apparent dissociation constant. On the other hand, total receptor densities using liver homogenates were lower for DR0.5 cell homogenates and DR3 cell homogenates compared to control rats.

Changes in percentage specific binding of ^{125}I-EGF with increasing concentration of unlabeled EGF were tested in the presence and absence of various concentrations of nonionic detergent Brij 35, using liver homogenates from control and DR0.5 rats, because of Dunn and Hubbard's (9) suggestion that Brij 35 unmasked internalized receptors. In the present studies, however, the addition of Brij 35 did not increase EGF binding to either the control or the DR0.5 cell homogenates at any concentration of EGF (tracer, 20, 40, 80 nM). Brij 35 rather decreased the binding of tracer concentration of ^{125}I-EGF by the receptors from control rat livers (data not shown).

Acid-Washing Method

To eliminate the potential effects of preadministered EGF on the binding assay, washing of the receptor-bound EGF prior to the binding assay may be required. Figure 2 shows the time courses of the release of the cell-associated EGF during the exposure of the cells to a pH 3.1 buffer and a pH 5.2 buffer. In both cases, a rapid and complete dissociation of EGF from the cell surface receptors occurred within 1–2 min. Further, the cells exposed to the pH 5.2 buffer retained binding activity comparable to that of control cells (Fig. 3). The binding parameters [Eq. (1)] were n = 0.07 pmol/mg of cellular protein, K_d = 3.1 nM, and α = 0.0027 ml/mg of cellular protein for control cells and n = 0.057 pmol/mg of cellular protein, K_d = 2.5 nM, and α = 0.0024 pmol/mg of cellular protein for acid-exposed cells.

Digitonin Treatment of Cells

Figure 4 shows the Scatchard plots for EGF binding to the cells with and without digitonin treatment. Digitonin-

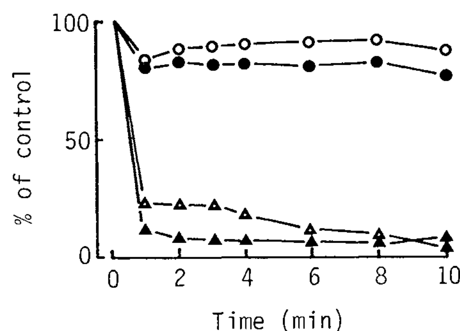

Fig. 2. Time courses of the release of the cell-associated ^{125}I-EGF by washing with either pH 3.1 (open symbols) or pH 5.2 buffer (filled symbols). Isolated hepatocytes were incubated with the tracer concentration of ^{125}I-EGF for 20 min at 37°C (○, ●) or for 1 hr at 0°C (△, ▲). Thereafter, the cells were exposed to the acid buffer, and the radioactivities released from the cells were determined. Values are shown as the percentage of the control value, which was determined prior to the acid washing. The data points are the means of two determinations.

treated cells were also washed with the pH 5.2 buffer. The resultant binding parameters in control cells and 100 n*M* EGF treated cells are summarized in Table II. Digitonin treatment increased the dissociation constant (K_d) in both the control and the EGF-treated cells. Further, digitonin treatment had little effect on the binding capacity in control cells, but remarkably increased binding in the EGF-treated cells.

DISCUSSION

RME involves the process of binding of ligands with cell-surface receptors, formation of coated pits, internalization of ligand–receptor complexes, degradation of ligands, and receptor recycling or degradation (22,23). EGF is taken up by the rat hepatocytes by RME, but the fate of the internalized receptors is not clear yet (13,23).

We have previously performed a kinetic analysis of the

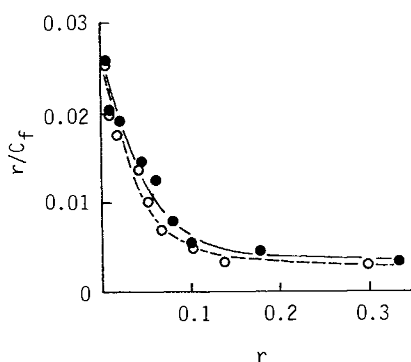

Fig. 3. Comparison of EGF binding by control hepatocytes (●) and by the cells treated by mild acid (○). Aliquots of the hepatocytes were exposed to the pH 5.2 buffer for 6 min at 0°C and washed twice with the standard buffer (pH 7.4). Then the EGF binding assays were performed. Aliquots of the cells were obtained from the same pool of hepatocytes that was used for the control experiments. Solid lines were calculated by an iterative nonlinear least-squares method based on Eq. (1). The data points are the means of two determinations.

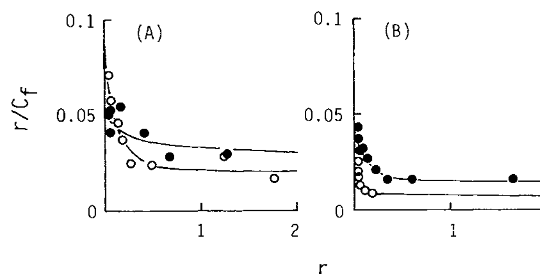

Fig. 4. Scatchard plots of the EGF binding of the digitonin-treated (●) and untreated (○) hepatocytes. Experiments were performed with control hepatocytes (A) and those incubated with 100 n*M* unlabeled EGF for 20 min at 37°C before digitonin treatment followed by mild acid washing (B). Solid lines were calculated by an iterative nonlinear least-squares method based on Eq. (1). The data points are the means of two determinations.

down-regulation of EGF receptors in rats *in vivo* (6), by measuring the rate of tissue EGF uptake. The present studies (Fig. 1, Table I) showed that the cell-surface receptor density in hepatocytes from rats sacrificed 30 min after a large EGF dose remarkably decreased compared to the control, with recovery of EGF cell surface binding 3 hr after the *in vivo* administration. This time course parallels that of the rate of cellular EGF uptake (6), supporting the hypothesis that the rate of cellular EGF uptake is a function of cell-surface receptor densities. Previous evidence showing that the time course for de novo EGF receptor synthesis is slow relative to the duration of the present experiment (8), and such showed receptor recycling (13). These results suggest that the recovery of cell-surface EGF receptors in the liver may be accounted for mainly by the recycling or recruitment of the internalized receptors.

Homogenates of the isolated hepatocytes were also used to detect the intracellular receptors. The total receptor density in the control-cell homogenate was comparable to the cell-surface receptor density in the control cells, indicating that there are few intracellular EGF receptors in control cells. However, the receptor densities of homogenates of DR0.5 and DR3 cells were much lower than those of DR0.5 and DR3 cells, respectively (Table I, Fig. 1). A possible reason for such smaller receptor densities is that EGF mol-

Table II. Parameter Values for EGF Binding to the Digitonin-Treated (+ Digitonin) and Untreated (− Digitonin) Hepatocytes[a]

	n (pmol/mg protein)	K_d (n*M*)	α (ml/mg protein)
Control cells			
− Digitonin	0.087	1.0	0.020
+ Digitonin	0.086	4.9	0.032
Cells incubated with 100 n*M* unlabeled EGF			
− Digitonin	0.017	0.9	0.009
+ Digitonin	0.089	3.4	0.015

[a] The data shown in Fig. 4 were fitted to Eq. (1) by an iterative nonlinear least-squares method. The values are the mean of two independent determinations.

ecules which were taken up by the liver after the preadministration may affect the binding measurements. Figure 5 shows a simulation of Scatchard plots with changing concentrations of preexisting EGF. Preexisting EGF (C_{pre}) should affect only the apparent dissociation constant, and not the binding capacity (n). In practice, however, the estimation of true binding capacity is difficult, because of reduced tracer binding.

In order to assess the density of intracellular receptors in the hepatocytes with mostly internalized EGF receptor, we initially tried to use Brij 35, a nonionic detergent, according to the method reported by Dunn and Hubbard (9). Brij 35 has been reported to allow access of EGF to intracellular receptors in the homogenates, although it may decrease the affinity of EGF to the receptors (9). In the present study, however, the binding capacity was not increased by Brij 35, at any Brij 35 concentration attempted. We do not know the reason for this discrepancy, but it may be related to differences in the Brij 35 batches.

Trypsin digestion was also reported to allow estimation of the density of intracellular insulin receptors (10). McClain and Olefsky (10) digested the cell-surface receptors with trypsin, and EGF binding was subsequently measured using solubilization by Triton X-100. However, in the present study, the cell-surface receptors were found to be resistant to trypsin treatment under the conditions used.

The use of digitonin for permeabilization has certain advantages. As the cell structure is maintained even after digitonin treatment, the same method (centrifugal filtration method) can be used as in determining the cell-surface receptor density for control cells, with minimal differences in conditions for the binding assay. Further, in combination with the pH 5.2 washing method, the effect of preexisting EGF molecules bound to intracellular receptors can be suppressed, as the dissociation of EGF from the receptors after the mild acid washing was rapid (Fig. 3), without any effect on receptor affinity (Fig. 4). Hence, digitonin treatment of cells previously exposed to EGF and subsequently washed with pH 5.2 buffer restored the high-affinity binding capacity to close to that of control cells (Fig. 4, Table II) and, thus, revealed the latent receptors (probably intracellular receptors). Slight increases in the K_d values after digitonin treatment may have resulted from changes in EGF receptor environment induced by the detergent, but this change in the K_d value should not affect estimates of the intracellular receptor densities.

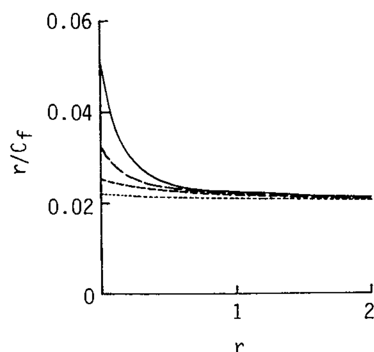

Fig. 5. Simulations of Scatchard plots for EGF binding. Simulations were performed based on Eq. (2), using the following parameter values: n = 0.1 pmol/mg of cellular protein, K_d = 3.0 nM, α = 0.02 ml/mg of cellular protein, and C_{pre} = 0 (——), 5 (—), 20 (---), and 100 nM (••••), respectively.

In conclusion, the combination of digitonin permeabilization and mild acid washing of the cells permits the assay of intracellular EGF receptors in rat hepatocytes.

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