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Fluorescent labeling of hormone receptors in viable cells: Preparation and properties of highly fluorescent derivatives of epidermal growth factor and insulin

(cell membranes/rhodamine/fluorescence photobleaching recovery/receptor mobility)

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ABSTRACT **Highly fluorescent analogs of insulin and epidermal growth factor were prepared by the covalent attachment of these peptides to α-lactalbumin molecules that were highly substituted (i.e., seven to one) with rhodamine molecules. The α-lactalbumin was specifically linked to the lysine residue of insulin or to the α-amino group of epidermal growth factor. The insulin derivative retained 1.15% of its potency in stimulating glucose oxidation in fat cells but retained about 8.3% of its binding affinity toward receptors. The epidermal growth factor derivative was completely active in binding to fibroblast receptors and 40% as potent as the native hormone in stimulating DNA synthesis. These highly fluorescent derivatives were suitable for the specific visual labeling of receptor sites in viable cells and for measuring the lateral mobilities of the receptor–hormone complexes by fluorescent photobleaching recovery techniques. By these methods it was shown that the hormone–receptor complexes can move laterally in the plane of the plasma membrane with a diffusion coefficient of (3–5) × 10^{-10} cm^2/sec.**

Fluorescence microscopy is used extensively for localizing different cell surface components such as surface antigens (1), lectin-binding sites (2), and toxin receptors (3). Recently, the fluorescent photobleaching recovery technique has made it possible to study quantitatively the dynamic properties of such fluorescently labeled molecules on viable cells under physiological conditions (4, 5).

Unfortunately, in only a few cases has it been possible to label hormone receptors with fluorescent analogs (6, 7). The number of hormone receptor sites per cell is generally so small (i.e., 10,000 to 40,000, at most) that a density of labeled receptors sufficiently high for microscopic detection is in theory difficult to achieve. Furthermore, polypeptide hormones have few potential sites for substitution with fluorophores without impairing the binding affinity of the hormone to its receptor. This makes it difficult or impossible to achieve by conventional methods the high fluorescence intensity that can be obtained by multiple fluorescent probe substitution. Extensive chemical modification of hormones may also cause gross changes in conformation, resulting in lowered affinity, specificity, and selectivity of binding to receptors on intact cells.

In the present studies these problems are overcome by coupling to peptide hormones a protein (α-lactalbumin) that has previously been highly substituted (i.e., seven to one) with rhodamine through single, uniquely specified hormone residues whose selective modification is known not to adversely alter the biological activity of the hormone. Thus, a highly fluorescent analog of α-lactalbumin can be coupled to the α-amino group of epidermal growth factor (EGF) or to the ε-amino group of the single lysyl residue of insulin. These derivatives retain partial biological activity, and they label receptors sufficiently well to permit localization by fluorescent microscopy and quantitation of lateral mobility by fluorescent photobleaching recovery methods.

MATERIALS AND METHODS

Materials. α-Lactalbumin, 5,5′-dithiobis(2-nitrobenzoic acid), dithiothreitol, iodoacetamide, 2-iodoacetic acid, citraconic anhydride, dansyl chloride, and *S*-acetylmercaptosuccinic anhydride were purchased from Sigma. Rhodamine (Rhod) isothiocyanate (tetramethyl form, hereafter referred to simply as rhodamine) was obtained from Research Organic Chemicals, 2,4-dinitrofluorobenzene and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MIB-NHS) from Pierce Products, and pork insulin from Eli Lilly. EGF was purified from male mouse submaxillary glands according to the procedure of Savage and Cohen (8). The following procedures were used with no modification: binding of ^{125}I-labeled insulin (^{125}I-insulin) to fat cells and liver membranes (9); [^{3}H]thymidine incorporation into DNA (10); conversion of D-[*U*-^{14}C]glucose to $^{14}CO_2$ (11); NH_2-terminal analysis of proteins with dansyl chloride (12).

Preparation of Rhod$_7$-α-Lactalbumin. To 5 ml of α-lactalbumin (3 mg/ml) in 0.2 M sodium carbonate buffer, pH 9.3, were added four aliquots of 0.3 ml of rhodamine isothiocyanate (10 mg/ml in dimethylformamide) over a period of 24 hr. The solution was then dialyzed extensively in the cold against distilled water and finally was lyophilized. The number of rhodamine groups incorporated was determined using ϵ_{545} = 80,000 M cm^{-1} (13). α-Lactalbumin concentration was determined by amino acid analysis. Approximately 7 mol of rhodamine were coupled per mol of α-lactalbumin.

Preparation of Citrac$_2$,$N^{\epsilon B29}$-MIB-Insulin. Dicitraconyl insulin (Citrac$_2$-insulin) was prepared as follows. A 30-ml solution of insulin (2 mg/ml) with trace quantities of ^{125}I-insulin in 0.01 M sodium carbonate was adjusted to pH 6.9. Two aliquots (5 μl each) of citraconic anhydride were added to the stirred protein solution over a period of 1 hr and the pH was kept at 6.8 to 6.9 by the addition of NaOH. The protein solution was then dialyzed at 7° against 4 liters of 0.05 M $NaHCO_3$

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Abbreviations: EGF, epidermal growth factors; MIB-NHS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; Rhod$_7$-α-lactalbumin, α-lactalbumin modified with an average of 7 mol rhodamine per mol of α-lactalbumin; Citrac$_2$-insulin, an insulin derivative in which both α-amino groups are citraconylated; Citrac$_2$,$N^{\epsilon B29}$-MIB-insulin, Citrac$_2$-insulin in which the lysine residue (B29) is modified to give an *N*-maleimidobenzoyl lysyl residue; $N^{\epsilon B29}$-Rhod-insulin, derivative in which a rhodamine molecule is covalently linked to the ε-amino group of lysine B29.

(adjusted to pH 7.0), for 16 hr. Under these conditions both α-amino groups were blocked and the ϵ-amino group of lysine residue B29 remained free, as was confirmed by end-group analysis and the complete absence of lysine on amino acid analysis following reaction of the derivative with 2,4-dinitrofluorobenzene and acid hydrolysis. The protein was further reacted with the bifunctional reagent MIB-NHS, to produce an insulin derivative that would react with sulfhydryl groups. Ten milliliters of $Citrac_2$-insulin (2 mg/ml in H_2O) was brought to pH 7.7 by the addition of NaOH cooled to 0° to 2° and three aliquots (0.4 ml each) of MIB-NHS (10 mg/ml in dimethylformamide) were added over a period of 20 min. The pH was maintained at 7.7 with NaOH. After 30 min the solution was adjusted to pH 6.0. After the reaction mixture was centrifuged to remove a fine precipitate produced during the reaction, it was dialyzed in the cold. The number of maleimidobenzoyl groups incorporated into the insulin molecule was determined by reacting a portion of the derivative with a 2-fold molar excess of reduced glutathione. The amount of unreacted glutathione was determined with 5,5'-dithiobis(2-nitrobenzoic acid) (14). Approximately 1 mol of the maleimidobenzoyl group was incorporated per mol of $Citrac_2$-insulin.

Coupling of $Citrac_2$,$N^{\epsilon B29}$-MIB-Insulin to Partially Reduced $Rhod_7$-α-Lactalbumin. To $Rhod_7$-α-lactalbumin (8.5 mg/ml in 0.2 M TrisCl, pH 7.0), a 0.10-ml aliquot of dithiothreitol solution (10 mM) was added (2-fold molar excess over the protein), and the reduction was allowed to proceed for 10 min. Then $Citrac_2$,$N^{\epsilon B29}$-MIB-insulin (1.2 mg, dissolved in 2.0 ml of 0.1 M TrisCl buffer, pH 7.0) was added. The coupling reaction proceeded for 1 hr. Iodoacetamide (1 mg) was then added and the reaction mixture was incubated for 10 min. It was then extensively dialyzed against water in the cold and lyophilized. No significant reduction of disulfide bonds of the insulin derivative under the coupling process took place, as confirmed by incubating $Citrac_2$,$N^{\epsilon B29}$-MIB-insulin with the same amount of dithiothreitol applied in the coupling process, followed by the addition of 2-iodoacetic acid. Only 0.1–0.2 mol of *S*-carboxymethylcysteine per mol of insulin was incorporated (as determined by acid hydrolysis and amino acid analysis).

The noncovalently bound insulin derivative was separated from the conjugate by gel filtration. The lyophilized material (15 mg) was dissolved in 0.7 ml of 0.1 M TrisCl, pH 8.5/7 M urea and loaded on a Sephadex G-50 column (1.5 × 60 cm) previously equilibrated with the same buffer. The column was developed at 50 ml/hr. The fractions corresponding to the first peak were pooled and dialyzed extensively against water.

The citraconyl groups were removed by incubating the derivative at pH 2.0 for 4 hr at room temperature. The pH was then brought to 9.3 and the protein solution was dialyzed extensively against water for 20 hr and then lyophilized.

The ratio of $^{125}I/A_{545}$ was used to determine the average mol ratio of insulin to $Rhod_7$-α-lactalbumin in the conjugate. This varied between 0.2 and 0.4 in various preparations.

Preparation of $N^{\epsilon B29}$-Rhod-Insulin. To 10 ml of $Citrac_2$-insulin (2 mg/ml) in 0.2 M sodium carbonate buffer, pH 9.3, three aliquots of 3 mg of rhodamine isothiocyanate in 0.3 ml of dimethylformamide were added at 3-hr intervals. The solution was incubated for an additional 3 hr. It was dialyzed in the cold against 0.1 M $NaHCO_3$ for 3 days and then dialyzed against distilled water for 2 more days before lyophilization. The blocking groups were then removed at acidic pH, as already described, and the derivative was purified on a DEAE-Sephadex column (A-25) using conditions identical to those applied by Lindsay and Shall (15) for the separation of partially acetylated insulin derivatives. The main reddish peak was dialyzed extensively against water and lyophilized. The derivative contained 1 mol of rhodamine per mol of insulin.

Preparation of $Rhod_7$-α-Lactalbumin-EGF Conjugate. The single α-amino group of EGF was modified with MIB-NHS to produce an EGF derivative that would react with sulfhydryl groups. To a solution of EGF (0.3 mg in 0.3 ml of cold 0.1 M phosphate buffer, pH 7.3) which contained trace quantities of ^{125}I-EGF, three aliquots (20 μl each) of MIB-NHS (10 mg/ml in dimethylformamide) were added over a period of 20 min. The solution was then centrifuged and loaded on a Sephadex G-15 column (15 × 1 cm). The elution was carried out with 0.2% bovine serum albumin. The EGF fractions were pooled and lyophilized.

Sulfhydryl groups were incorporated into $Rhod_7$-α-lactalbumin by successive succinylation of the remaining amino groups with *S*-acetylmercaptosuccinic anhydride. $Rhod_7$-α-lactalbumin (10 ml, 2 mg/ml) was brought to pH 7.0 and five aliquots (3 mg each) of solid *S*-acetylmercaptosuccinic anhydride were added over a period of 1 hr. The pH was kept at 7.0 to 7.3 with NaOH. The protein solution was then dialyzed extensively against water and lyophilized.

The coupling was performed by incubating the α-lactalbumin (0.1 mM) and EGF (0.05 mM) derivatives in 0.1 M TrisCl/50 mM NH_2OH (pH 7.0) for 20 min. Solid iodoacetamide was then added (final concentration, 0.5 mM) and the solution was dialyzed extensively against distilled water. Any EGF derivative not covalently bound to $Rhod_7$-α-lactalbumin was removed by using an Amicon–Diaflo cell (membrane PM-10). The conjugated derivative contained 0.3 mol of EGF per mol of $Rhod_7$-α-lactalbumin.

RESULTS AND DISCUSSION

Fig. 1 describes schematically the steps involved in the preparation of $Rhod_7$-α-lactalbumin-insulin conjugate, using the side chain of lysine B29 of insulin and the reduced disulfide bond I-VIII of α-lactalbumin for bridging both molecules. α-Lactalbumin is a particularly suitable inert protein for coupling to small polypeptides for the purposes described here. It is a small globular protein (molecular weight 14,500) and it contains many lysine residues, thus permitting the incorporation of a large number of fluorescent probes.

Certain information used here was crucial in the design and preparation of the $Rhod_7$-α-lactalbumin-insulin conjugate. Various studies of chemical modification of insulin had consistently shown that the ϵ-amino group of lysine B29 of insulin can be modified, even with bulky substituents, with little or no change in the biological activity of the hormone (16). In addition, we had recently observed that the binding of avidin to an insulin derivative that contains a biotinyl moiety covalently attached to lysine B29 does not destroy the biological potency or the receptor binding affinity of the complex. On the other hand, if the same complex is formed with an insulin derivative in which the biotinyl group is attached to the α-amino group of phenylalanine B1, both the binding affinity and biological potency are greatly reduced.‡ Thus, we concluded that the ϵ-amino group of lysine B29 would be the most suitable candidate for attaching a macromolecule to the hormone without adversely affecting its biological properties.

In addition, it has been observed (17) that the disulfide bond I-VIII of α-lactalbumin can be selectively reduced by low concentrations of dithiothreitol (2 to 5 mol equivalents). Essentially the same situation exists in $Rhod_7$-α-lactalbumin. Thus, given the coupling conditions used here, only two SH groups are available for attachment. In addition, the use of a relatively

‡ Y. Shechter, S. Jacobs, K.-J. Chang, and P. Cuatrecasas, unpublished data.

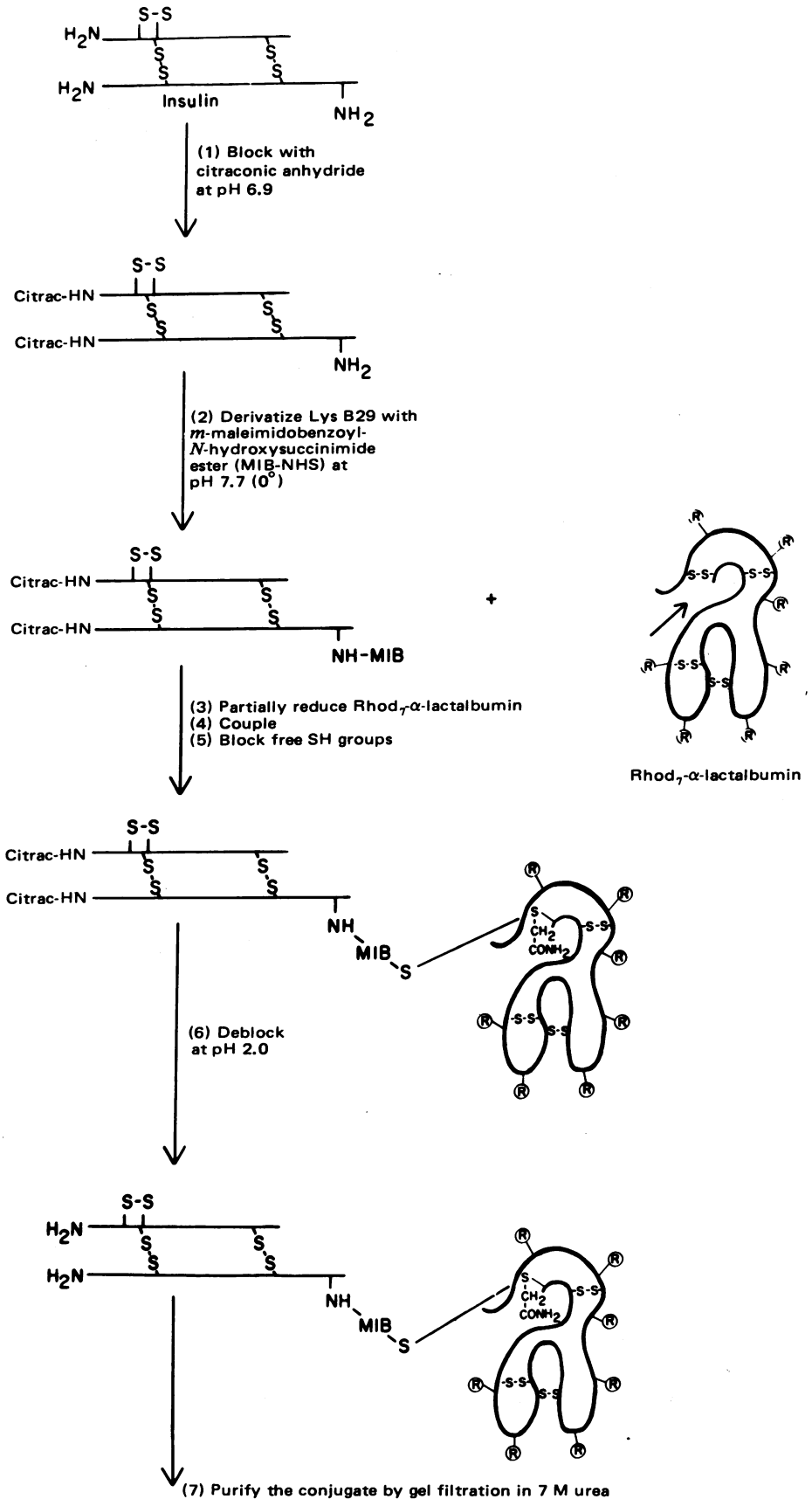

FIG. 1. Steps involved in the preparation of $Rhod_7$-α-lactalbumin-insulin conjugate.

Table 1. Binding and biological activity of various fluorescent analogs of insulin and EGF

Hormone or derivative	Binding affinity to cells*		Biological activity†	
	50% ^{125}I-hormone displacement, ng/ml	Relative binding potency,‡ %	ED_{50},§ ng/ml	Relative activity, %
Insulin	40	100	0.15	100
$N^{\epsilon B29}$-Rhod-insulin	45	89	0.20	75
Rhod$_7$-α-lactalbumin-insulin	480	8.3	13	1.15
EGF	8.2	100	0.13	100
Rhod$_7$-α-lactalbumin-EGF	8.2	100	0.33	40

* For insulin experiments, isolated rat adipocytes (1.6×10^6 cells) and ^{125}I-insulin (1 ng/ml) were incubated with various concentrations of native insulin or its derivatives in 0.25 ml of Krebs–Ringer bicarbonate buffer, pH 7.4/1% bovine serum albumin for 45 min at 24°. Insulin binding was determined by floating adipocytes over silicone oil in a Beckman Microfuge. For EGF experiments, a suspension of confluent 3T3 mouse fibroblasts (0.2 ml) in Krebs–Ringer bicarbonate buffer, pH 7.4/1% albumin (about 2.2×10^6 cells per ml) was incubated at 15° with 27 pg of ^{125}I-EGF and with various concentrations of native EGF or its derivatives for 2 hr. The amount of ^{125}I-EGF bound was determined using EAWP Millipore filters (9).

† Experimental values obtained from Fig. 2.

‡ $100 \times IC_{50}$ of native hormone ÷ IC_{50} of derivative, in which IC_{50} is the concentration of native hormone or derivative that inhibits 50% of the ^{125}I-hormone binding.

§ Concentration of insulin or EGF that produced half-maximal stimulation.

high mol excess of Rhod$_7$-α-lactalbumin derivative relative to the insulin derivative was applied in an attempt to eliminate the possible formation of a conjugate with a ratio of insulin to Rhod$_7$-α-lactalbumin of more than one.

The method of conjugation used is unidirectional and therefore precludes the formation of products of polymerized insulin or α-lactalbumin.

Special precautions were taken to remove any unbound insulin derivative from the conjugate. For this purpose a step of gel filtration on a Sephadex G-50 column is used in the presence of 7 M urea under conditions in which insulin exists exclusively in the monomeric state. In a control experiment mixed native insulin and Rhod$_7$-α-lactalbumin were fully resolved under identical conditions. In addition, rechromatography of the purified conjugate did not change its biological specific activity, thus indicating the absence of unbound insulin.

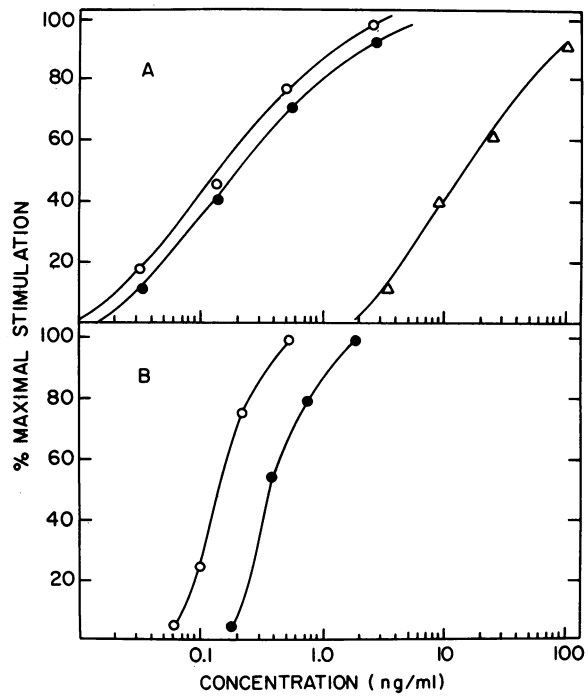

FIG. 2. Biological activities of fluorescent derivatives of insulin and EGF. (*A*) Isolated rat adipocytes (10^5 cells) were incubated for 90 min at 37° in Krebs–Ringer bicarbonate buffer/1% albumin, pH 7.4, with 0.2 mM D-[*U*-^{14}C]glucose (5 Ci/mmol) and various concentrations of native insulin (○), $N^{\epsilon B29}$-Rhod-insulin (●), and Rhod$_7$-α-lactalbumin-insulin conjugate (△). (*B*) 3T3 mouse fibroblasts were grown to confluence (approximately 3×10^5 cells, 100 μg of protein) in 1.5-cm wells and then incubated with various concentrations of native EGF (○) or Rhod$_7$-α-lactalbumin-EGF conjugate (●) for 20 hr. The cells were then incubated with 0.15 μM thymidine (1.0 μCi/ml) for 1 hr. Results are expressed as percent of maximal stimulation (average of three replicate determinations).

The single amino group of EGF can be modified with no change in the biological potency of the hormone (unpublished observation). Hence, this group is used to covalently attach the hormone to Rhod$_7$-α-lactalbumin. In an alternative approach, SH groups are introduced into Rhod$_7$-α-lactalbumin by succinylation of the derivative with *S*-acetylmercaptosuccinic anhydride. Also, excess quantities of the α-lactalbumin derivative relative to the EGF derivative are applied in this coupling step to approximate stoichiometric substitution.

The conjugated derivatives of both hormones contain 0.2–0.4 mol of insulin or of EGF per mol of Rhod$_7$-α-lactalbumin, indicating that some free Rhod$_7$-α-lactalbumin is present in the preparation. The presence of this material is not disadvantageous because it is not adsorbed by the fibroblasts and is subsequently readily removed by washing. The fibroblasts in effect serve as affinity probes due to their selective affinity toward the conjugates.

The binding affinities and the biological potencies of the various conjugates prepared are summarized in Table 1 and Fig. 2. As can be seen, the Rhod$_7$-α-lactalbumin conjugate of insulin is only 1.15% as active as native insulin in stimulating glucose oxidation in fat cells. However, its binding affinity, 8.3%, is greater. Although the binding of the hormone is significantly perturbed on conjugation, its affinity and specificity are still sufficiently great to ensure highly specific labeling of the receptor sites of fibroblasts. The Rhod$_7$-α-lactalbumin conjugate of EGF retains its full binding capacity and 40% of its ability to stimulate DNA synthesis (Table 1 and Fig. 2).

Employing these fluorescent derivatives of insulin and EGF, and using the fluorescence photobleaching recovery method (4, 5, 16), we have been able to show that the fluorescence emitted from the labeled cells is above the background fluorescence. More than 60% of the total fluorescence could be displaced by incubating cells with native hormone at 1 μg/ml. The remaining background fluorescence was immobile on the experimental time scale of the bleaching experiment (4, 5). It is therefore possible to use these probes for studying the mobility of the hormone–receptor complexes. In fluorescence photo-

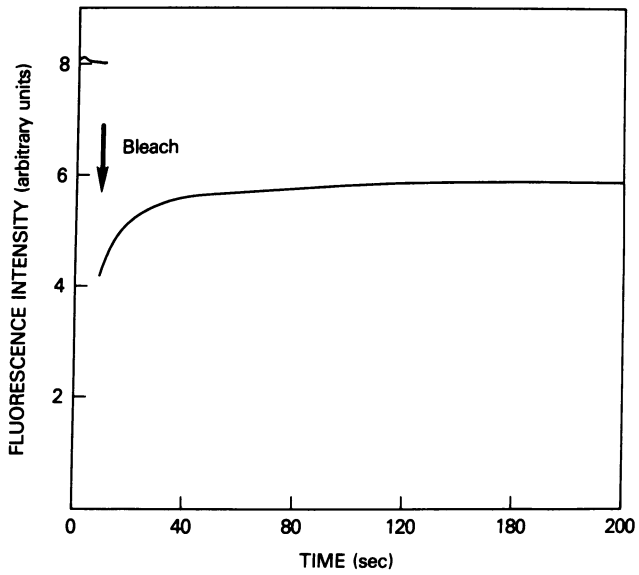

FIG. 3. Fluorescent recovery curve of 3T3 cells labeled with Rhod$_7$-α-lactalbumin-EGF conjugate at 23° for 20 min. Full details about the fluorescent photobleaching method are described in refs. 4, 5, and 18.

bleaching recovery a brief laser pulse photobleaches the fluorescence from a small area on the cell and then the rate at which fresh fluorophores move laterally into the bleached area is monitored (5). Cells labeled with $N^{\epsilon B29}$-Rhod-insulin were not adequate for fluorescence photobleaching recovery experiments, despite the good retention of biological and binding activities of this derivative (Table 1). This is mainly due to the high degree of background fluorescence.

A typical recovery curve is illustrated in Fig. 3. In this experiment 3T3 cells were labeled with Rhod$_7$-α-lactalbumin-EGF and then the fluorescence photobleaching recovery experiments were performed. From this and similar experiments performed on cells labeled either with Rhod$_7$-α-lactalbumin-EGF or Rhod$_7$-α-lactalbumin-insulin, we have been able to show that both hormone–receptor complexes *are mobile*, with diffusion coefficients in the range of (3–5) $\times 10^{-10}$ cm^2/sec. These findings are compatible with the previously proposed mobile receptor hypothesis of hormone receptor action (19). Full details of these studies, as well as those that utilize these analogs to visualize the qualitative pattern and redistribution of surface receptors by fluorescence microscopy, will be published elsewhere.

In conclusion, the present work describes the preparation of highly fluorescent derivatives of insulin and EGF that retain partial biological activity. These derivatives can be used to label and detect cell surface receptors by fluorescence microscopy, and the lateral diffusion of the hormone–receptor complexes so labeled has been ascertained. We hope that pursuing such studies in depth will extend our understanding of the dynamic properties and biochemical events that follow the initial hormone–receptor interaction.

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1. Taylor, R. B., Duffus, W. P. H., Raff, M. C. & de Petris, S. (1971) *Nature New Biol.* **233**, 225–229.
2. Edelman, G. M. (1976) *Science* **192**, 218–226.
3. Craig, S. W. & Cuatrecasas, P. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3844–3848.
4. Schlessinger, J., Koppel, D. E., Axelrod, D., Jacobson, K., Webb, W. W. & Elson, E. L. (1973) *Proc. Natl. Acad. Sci. USA* **73**, 2409–2413.
5. Koppel, P. E., Axelrod, D., Schlessinger, J., Elson, E. L. & Webb, W. W. (1976) *Biophys. J.* **16**, 1055–1069.
6. Varga, G. M., Moellmann, G., Fritch, P., Godawska, E. & Lerner, A. B. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 559–562.
7. Malamed, E., Lahav, M. & Atlas, D. (1976) *Nature* **261**, 420–421.
8. Savage, C. R. & Cohen, S. (1972) *J. Biol. Chem.* **247**, 7609–7611.
9. Chang, K.-J. & Cuatrecasas, P. (1974) *J. Biol. Chem.* **249**, 3170–3180.
10. Hollenberg, M. D. & Cuatrecasas, P. (1975) *J. Biol. Chem.* **250**, 3845–3853.
11. Rodbell, M. (1964) *J. Biol. Chem.* **239**, 375–380.
12. Gray, W. R. (1967) in *Methods in Enzymology*, ed. Hirs, C. W. H. (Academic, New York), Vol. 11, pp. 139–151.
13. Goldman, M. (1968) *Fluorescent Antibody Methods* (Academic, New York).
14. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77.
15. Lindsay, D. G. & Shall, S. (1971) *Biochem. J.* **121**, 737–745.
16. Blundell, T., Dodson, G., Hodgkin, D. & Mercola, D. (1972) *Adv. Protein Chem.* **26**, 279–402.
17. Shechter, Y., Patchornik, A. & Burstein, Y. (1973) *Biochemistry* **12**, 3407–3413.
18. Axelrod, D., Koppel, D. E., Schlessinger, J., Elson, E. & Webb, W. W. (1976) *Biophys. J.* **16**, 1055–1069.
19. Cuatrecasas, P. (1974) *Annu. Rev. Biochem.* **43**, 169–214.