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THE STABILITY IN VARIOUS DETERGENTS OF TRANSFERRIN-TRANSFERRIN RECEPTOR COMPLEXES FROM RETICULOCYTE PLASMA MEMBRANES

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Summary

Transferrin-membrane protein complexes were solubilized either with 0.4% sodium dodecyl sulfate (SDS), 1% Triton X-100 or 0.5% sulfobetaine 3-14 from the plasma membranes of rabbit reticulocytes previously labeled with ^{125}I and then incubated with ^{131}I-labeled transferrin. When the solubilized membranes were analyzed by gel filtration fractionation, marked variation in the preservation of transferrin-transferrin receptor interaction was noted between the three detergents. After SDS solubilization, more than 80% of the ^{131}I-labeled transferrin remained associated with membrane proteins with apparent molecular weight of the transferrin-receptor complexes of 1 400 000 and 240 000. In contrast, after Triton X-100 solubilization only 40% of the transferrin was still complexed to membrane proteins with an apparent molecular weight of the complex of 450 000. Dissociation of transferrin from its receptor was most marked following sulfobetaine solubilization, with less than 30% of the transferrin still complexed.

Following gel filtration ^{131}I-labeled transferrin-^{125}I-labeled membrane protein complexes were immunoprecipitated with goat specific anti-rabbit transferrin antibodies. The immunoprecipitates were analyzed under stringent dissociating conditions by two SDS-polyacrylamide gel electrophoretic techniques. In a linear 5—25% polyacrylamide gradient the ^{125}I-labeled receptor obtained after membrane solubilization with all three detergents had an apparent

Abbreviation: SDS, sodium dodecyl sulfate.

molecular weight of 80 000. In contrast, in a different system using 10% polyacrylamide gel two ^{125}I-labeled receptor components were detected with apparent molecular weights of 90 000 and 80 000. These results demonstrate that estimates of the molecular weight of the transferrin receptor depend on the conditions of electrophoresis and suggest that the transferrin receptor is partially modified, perhaps by glycosylation.

Introduction

The first step in the delivery or iron to the developing erythroid cell is the binding of transferrin to specific sites on the cell membrane [1—3]. Identification of the membrane proteins which comprise the transferrin receptor would allow definition of the molecular basis of the interaction of transferrin with its receptor. Many attempts have been made to identify and isolate the transferrin binding site or receptor [4—14], giving rise to what appear to be several different moieties as judged by molecular weight. We have recently identified a membrane protein that remains associated with transferrin after solubilization of reticulocyte plasma membrane with the ionic detergent SDS under mild conditions [14]. In this report three different detergents were used to solubilize reticulocyte membranes previously labeled with ^{125}I. Specificity of isolation of the transferrin-receptor complex was increased by immunoprecipitation with specific anti-transferrin antibodies. Although the stability of the transferrin-receptor complex varied in the various detergents, the electrophoretic properties of the receptor proteins were similar. The apparent molecular weight of the receptor protein was dependent on the conditions of SDS-polyacrylamide gel electrophoresis, a characteristic previously described for membrane glycoproteins [15].

Materials and Methods

Preparation of rabbit reticulocyte plasma membranes and ^{131}I-labeled transferrin were as described [14].

Iodination of reticulocyte membrane proteins with ^{125}I. Washed reticulocytes were depleted of transferrin as described by Hemmaplardh and Morgan [16]. Cells were subsequently labeled with ^{125}I by the iodine monochloride method [17] with the following modifications. 1 vol. of cells was mixed with an equal volume of phosphate-buffered saline and 0.1 vol. of alkaline/glycine buffer. Cells were warmed to 37°C and 0.1 vol. ^{125}I and stock iodine monochloride solution in alkaline/glycine buffer was added. Cells were incubated 20 min at 37°C and washed three times with phosphate-buffered saline. No lysis of cells occurred during the procedure. In preliminary studies this method of iodination produced a greater degree of labeling than with the lactoperoxidase method [18]. Subsequent polyacrylamide gel electrophoretic analysis of the membrane proteins from ^{125}I-labeled reticulocytes showed that the most extensively labeled proteins were bands 1, 2 and 3 (using the nomenclature of Fairbanks et al. [19]).

Preparation of ^{131}I-labeled transferrin-^{125}I-labeled membrane complexes. An

aliquot of ^{125}I-labeled cells was incubated with ^{131}I-labeled diferric transferrin for 20 min at 37°C. Cells were washed three times with phosphate-buffered saline and membranes prepared as described [14]. Membranes (2 mg protein/ml) were made 0.4% in SDS, incubated at 37°C for 5 min, chilled, and applied to a Biogel A-5 column, 1.5 × 50 cm, equilibrated with 20 ideal mosM (imosM) Tris-HCl (pH 7.6)/0.005% SDS. The addition of SDS to the column elution buffer prevented aggregation of high molecular weight proteins. In control studies 0.005% SDS by itself neither lysed cells, solubilized membrane proteins not interferred in immunoprecipitation of the transferrin-receptor complex.

Solubilization of membranes with Triton X-100 and sulfobetaine 3-14. Membranes (2 mg protein/ml) in 20 imosM Tris-HCl (pH 7.6) were made 1% in Triton X-100 or 0.5% sulfobetaine 3-14, kept at 4°C for 5 min and centrifuged at 25 000 × *g* for 20 min. The supernatants were then subjected to gel filtration under the same conditions as for SDS-solubilized membranes.

Precipitation of transferrin receptor complexes by anti-rabbit transferrin IgG. Antibodies against rabbit transferrin were raised in goats and the IgG fraction purified by chromatography on DEAE-Sephadex and Biogel A-1.5 [20]. Pooled fractions from Biogel A-5 fractionation of solubilized plasma membranes containing transferrin-receptor complex were concentrated by ultrafiltration and reacted with anti-transferrin antibody for 15 min at 4°C. 20 μg carrier transferrin were added and incubation continued for 45 min at 4°C. The immunoprecipitate was pelleted at 1000 × *g* and washed 4 times with phosphate-buffered saline. To control for non-specific trapping or absorption of ^{125}I-labeled membrane proteins to the immunoprecipitate, transferrin-free ^{125}I-labeled membranes were subjected to the process of solubilization, gel filtration and immunoprecipitation.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis on 5—25% polyacrylamide gradient slab gels (0.15 × 11 × 14 cm) was performed according to the method of Laemmli [21]. Electrophoresis was continued for an additional 45 min at 35 mA constant current after the tracking dye reached the bottom of the gel. This procedure produces tight, sharply defined, protein bands on subsequent staining with Coomassie blue. Additionally, to allow comparison with other studies [8—10,12,13] analysis was also conducted with 10% polyacrylamide gels using the method of Fairbanks et al. [19]. Aliquots from the same transferrin-receptor immunoprecipitate were applied to both gel systems. Radioactivity was determined by slicing the gels in 2-mm fractions and measuring radioactivity in a 1185 Searle automatic gamma counter, correcting for the crossover of ^{131}I radioactivity into the ^{125}I channel.

Isoelectrofocusing in polyacrylamide gels was performed as previously described [14] using a pH gradient of 3.5—10. Samples were focused for 2000 V · h.

Materials

Biogel A-5, 200—400 mesh, SDS and reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories. Protein standards for gel filtration and electrophoresis were from Pharmacia Chemical Company. ^{125}I and ^{131}I were products of New England Nuclear Corp. Sulfobetaine 3-14 and Triton X-100 were products of Calbiochem-Behring Corp. Other reagents were analytical grade or better.

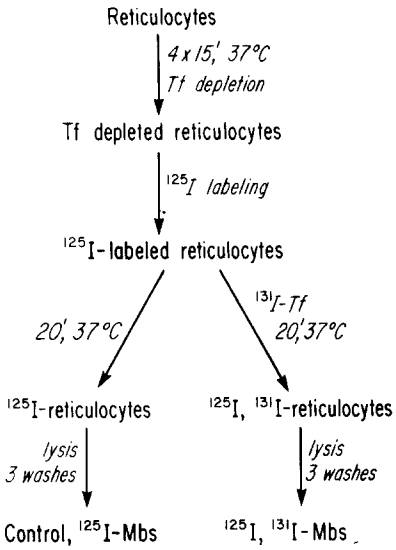
Results

The protocol used for the preparation of labeled membranes is shown in Fig. 1. In all studies ^{125}I was used to label reticulocyte membrane proteins and ^{131}I to label transferrin. To prevent differences in the extent of labeling between control and test membranes, an aliquot of cells to be used for control was taken after [^{125}I]iodine labeling. These control cells were used to prepare ^{125}I-labeled membrane without additional incubation with ^{131}I-labeled transferrin.

Transferrin depletion of the cells before iodination was necessary to avoid iodination of transferrin bound to reticulocytes. The effectiveness of the depletion process was controlled by measuring the release of ^{131}I-labeled transferrin from reticulocytes. Over 90% of the radioactivity was released from the cells after four consecutive washes.

Gel filtration of solubilized (^{131}I,^{125}I)-labeled membrabes. We have previously demonstrated that solubilization of reticulocyte membranes with SDS under mild conditions preserves the interaction between transferrin and a membrane protein [14]. We wanted to compare the stability of the transferrin-receptor complex in the presence of detergents other than SDS, as alterations of the stability of the complex in the presence of various detergents could provide insight into the nature of the interaction between transferrin and its receptor. Additionally, polyacrylamide gel electrophoretic analysis of the transferrin-receptor complex isolated after solubilization with various detergents would indicate whether similar or different membrane proteins remain associated with transferrin.

Fig. 2 shows the gel filtration pattern of membranes solubilized either with SDS, Triton X-100 or sulfobetaine 3-14. The ^{131}I-labeled transferrin profile of SDS-solubilized membranes (Fig. 2a) shows two major peaks at 1 400 000 and 240 000 apparent molecular weight (Peak I and Peak II as in Ref. 14) with only a small peak of free transferrin. As described previously [14] the transferrin in Peak I represents transferrin which has not yet donated its iron to the plasma membrane while the transferrin in Peak II is partially depleted of iron but not yet released from the membrane. The apparent molecular weights of Peaks I and II are both slightly higher, in the present study using Biogel A-5 in the presence of 0.005% SDS, than those previously reported using Biogel A-1.5 [14]. More than 80% of the transferrin remains associated with the membrane proteins found in Peaks I and II as reported previously [14]. The amount of free transferrin increases as membranes are stored at —20°C for periods of 1 week or longer. Gel filtration of membranes solubilized by Triton X-100 (Fig. 2b) shows two peaks of ^{131}I-labeled transferrin radioactivity with apparent molecular weights of 450 000 and 78 000, the latter corresponding to free transferrin. In contrast to SDS-solubilized material, after Triton solubilization the bound transferrin represents only approximately 40% of the total transferrin radioactivity. Therefore, although Triton X-100 solubilizes over 95% of the membrane-associated transferrin, less than half of this transferrin remains associated with membrane protein(s). This dissociation of transferrin from its presumed receptor is even more noticeable when the zwitterionic detergent sulfobetaine 3-14 is used as a solubilizing agent (Fig. 2c). With this detergent the bound



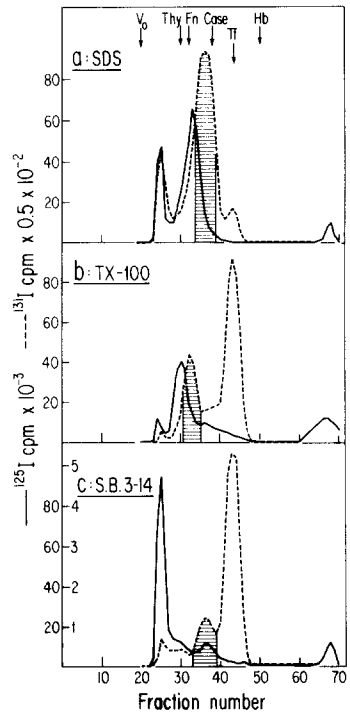


Fig. 1. Flow-sheet for the preparation of (^{125}I,^{131}I)-labeled membranes and of control ^{125}I-labeled membranes. Reticulocytes were depleted of endogenous transferrin (Tf) and labeled with ^{125}I. After extensive dialysis to eliminate non-bound ^{125}I the sample was divided into aliquots. One aliquot was kept as a control and the other incubated with ^{131}I-labeled transferrin as described in Materials and Methods. Cells were lysed and membranes prepared as described [14]. Mbs, membranes.

Fig. 2. Gel filtration of labeled membrane solubilized with SDS, Triton X-100 (TX-100) or sulfobetaine 3-14 (S.B. 3-14). (^{125}I,^{131}I)-labeled membranes were solubilized with either 0.4% SDS (a), 1% Triton X-100 (b), or 0.5% sulfobetaine 3-14 (c). The solubilized proteins were loaded onto Biogel A-5 columns eluted with 20 imosM Tris-HCl (pH 7.6)/0.005% SDS, and fractions of 1.2 ml assayed for ^{125}I (———) and ^{131}I (- - - - -) radioactivity. Control ^{125}I-labeled membranes were submitted to the same procedure. The pattern of ^{125}I radioactivity from the control membranes was indistinguishable from the ^{125}I radioactivity from the (^{125}I,^{131}I)-labeled membranes (data not shown). Elution of protein standards, thyroglobulin (Thy, 669 000), ferritin (Fn, 440 000), catalase (Case, 232 000), transferrin (Tf, 78 000) and hemoglobin (Hb 62 000) are indicated in the figure as is the void volume (V_0). Fractions included in the shaded areas, representing the transferrin-receptor complexes, were subsequently pooled and immunoprecipitated.

transferrin peak accounts for less than 30% of the ^{131}I radioactivity, although the location of the transferrin-receptor complex is similar to that observed with SDS solubilization. These results indicate that solubilization with SDS provides a marked advantage over the non-ionic detergent Triton X-100 and the zwitterionic detergent sulfobetaine 3-14 in preserving the interaction of transferrin with its putative receptor.

Immunoprecipitation of the transferrin-receptor complex. Transferrin-receptor complexes pooled from the gel filtration fractions (shaded areas in Fig. 2) were immunoprecipitated with goat specific anti-rabbit transferrin immunoglobulins. This precipitation allows selection of membrane proteins associated

with transferrin from an heterogeneous population of membrane proteins [14]. About 66% of the ^{131}I-labeled transferrin in the pooled fractions was immunoprecipitable. Of the ^{125}I radioactivity in the same fractions 2.8—3.2% was precipitated, representing the ^{125}I-labeled membrane proteins specifically associated with transferrin. To control for nonspecific precipitation of membrane proteins, immunoprecipitation was also performed using similar fractions derived from the gel filtration of transferrin-depleted ^{125}I-labeled membranes. Only small amounts of ^{125}I radioactivity were immunoprecipitated from the control membranes. As will be shown later, the ^{125}I radioactivity in the immunoprecipitation of the control membranes was not associated with any particular protein.

SDS-polyacrylamide gel electrophoresis of the transferrin-receptor immunoprecipitates. To identify the constituents of the putative transferrin-receptor complexes the immunoprecipitates were analyzed by two different SDS-polyacrylamide gel electrophoresis systems under dissociating conditions [19,21]. Analysis by two systems provides a direct comparison between the estimation of the molecular weight of the putative receptor previously reported by us using the gel system of Laemmli [14] and the estimate derived by others using the gel system of Fairbanks [8,9,11—13]. This is an important and necessary comparison as proteins may have different migration properties depending on the electrophoretic system used for analysis [15,22]. The results of these comparisons are shown in Fig. 3. Using the Laemmli system (Fig. 3a, b and c), the immunoprecipitates of the transferrin-receptor complexes derived after membrane solubilization by all three detergents yielded similar patterns, with a very prominent peak of ^{125}I radioactivity at an apparent molecular weight of 80 000 with some slight trailing of radioactivity into the 90 000 molecular weight region. This ^{125}I-labeled membrane protein migrates to the same position as the ^{131}I-labeled transferrin. Radioactivity in the region above molecular weight 90 000 was insignificant and not clearly distinguishable from immunoprecipitates of control membranes. When a linear 10% polyacrylamide Laemmli gel was used instead of the 5—25% gradient gel, the putative receptor migrated as a broader band encompassing a region between 92 000 and 80 000 apparent molecular weight (data not shown). Using the Fairbanks gel system the membrane receptor migrated in a wider area with two distinct peaks at apparent molecular weights 90 000 and 80 000 (Fig. 3d, e and f). A small peak of ^{125}I radioactivity in the region of apparent molecular weight 190 000 was variably seen. With neither system did electrophoresis of immunoprecipitates of control membranes (Fig. 3, dotted lines) give distinctive peaks of ^{131}I radioactivity in the region corresponding to transferrin or in the 80 000—90 000 molecular weight range; hence, the reticulocytes were both effectively depleted of transferrin and the distinct ^{125}I radioactive peaks could not be attributed to nonspecific absorption to immunoprecipitates.

Isoelectrofocusing of the purified transferrin receptor. Purified transferrin receptor was obtained from immunoprecipitated ^{131}I-labeled transferrin-^{125}I-labeled receptor complex. To dissociate the receptor from immunoprecipitated transferrin the washed precipitate was resuspended in a small volume (generally 0.1—0.2 ml) of 20 imosM Tris-HCl (pH 7.6) containing 0.5% sulfobetaine 3-14. After incubation for 5 min at 37°C the immunoprecipitate was re-pelleted by

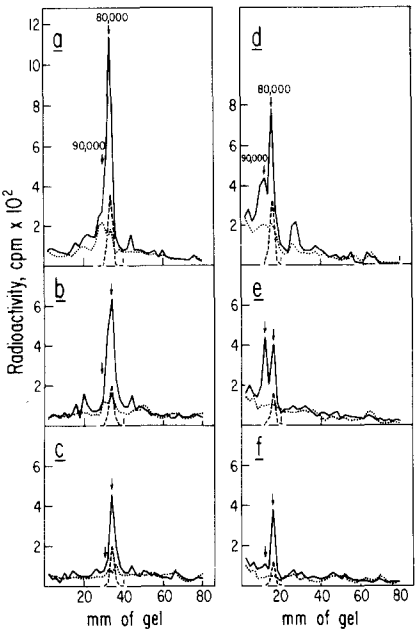


Fig. 3. SDS-polyacrylamide gel electrophoresis of transferrin-transferrin receptor immunoprecipitates. Transferrin-receptor complexes (Fig. 2, shaded areas) were immunoprecipitated with goat anti-rabbit transferrin antibodies. Samples were divided into aliquots and electrophoresed either in 5—25% polyacrylamide gradient SDS gels using the buffer systems of Laemmli [21] (a, b and c) or in 10% polyacrylamide SDS gels using the system of Fairbanks et al. [19] (d, e and f). Panels a and d represent complexes from membranes solubilized with 0.4% SDS; b and e represent complexes from Triton X-100 solubilization; and c and f represent complexes from sulfobetaine solubilization. After electrophoresis, gels were sliced into 2-mm fractions and assayed for ^{125}I (———) and ^{131}I (- - - - -) radioactivity from (^{125}I,^{131}I)-labeled membranes. The ^{125}I radioactivity (· · · · · ·) from control membranes is also shown. The migration of standard proteins for the polyacrylamide gels in panels a, b and c were phosphorylase *b* (M_r 94 000), 31 mm; bovine serum albumin (M_r 67 000), 39 mm; ovoalbumin (M_r 43 000), 48 mm; trypsin inhibitor (M_r 20 100), 65 mm; whereas the migration of the same standards in the polyacrylamide gels in panels d, e, and f were 12, 19, 29 and 52 mm, respectively. The major membrane, protein i.e., ^{125}I radioactivity, coincided with the ^{131}I-labeled transferrin radioactivity and had an apparent molecular weight of 80 000 as determined by the protein standards.

centrifugation at 25 000 × *g* for 30 min and the clear supernatant carefully collected. By this procedure 40—60% of the ^{125}I-labeled membrane proteins could be dissociated from the transferrin in the immunoprecipitate with no release of ^{131}I-labeled transferrin from the antitransferrin antibody. On isoelectrofocusing the purified receptor has a major peak of radioactivity with a p*I* equal to 6.98 ± 0.11 (mean ±S.D. of five determinations). This value correlates well with the p*I* of 6.4 previously described for a transferrin-receptor complex [14] and p*I* 6.6 for a putative placental transferrin receptor [13].

Discussion

The studies reported here allow us to clearly identify the membrane receptor to which transferrin binds and also to resolve differences in previously reported molecular weight characteristics of the receptor. Identification of the

receptor was achieved by an experimental design which introduced a ^{125}I label onto reticulocyte membrane proteins. The label allowed continued identification of the plasma membrane transferrin receptor after detergent solubilization of the membrane, gel filtration fractionation, and immunoprecipitation of the transferrin-receptor complex. The apparent disparities in size estimates of the receptor were resolved by observing the behavior of the putative ^{125}I-labeled transferrin receptor under different electrophoretic conditions.

Two possible limitations of the methods used include: 1, failure to label with ^{125}I the proteins involved in the receptor complex; and 2, dissociation of a receptor protein from the complex during membrane solubilization or immunoprecipitation. In either instance the methods would fail to detect a protein as part of the transferrin receptor. Iodination of a membrane transferrin binding site or receptor required depletion of transferrin bound to the reticulocyte to allow full exposure of the binding site to the labeling reagents. Clearly the transferrin-depleted membranes on immunoprecipitation with antitransferrin did not contain ^{125}I-labeled transferrin (Fig. 3). Witt and Woodworth [12] have reported similar results recently. Iodination of the binding site did not impede subsequent transferrin binding to labeled reticulocytes. Proof that the receptor site was iodinated derives from the co-precipitation by anti-transferrin antibodies of ^{125}I-labeled proteins along with transferrin only when transferrin was allowed to bind to reticulocytes after the ^{125}I labeling.

SDS membrane-solubilization was markedly superior to the other detergents in maintaining the interaction of transferrin and its receptor. The molecular basis for the apparent difference in complex stability is not clear. Triton X-100 binds to hydrophobic domains of glycoproteins [23]; the large dissociative activity of Triton suggests the importance of hydrophobic regions in the transferrin-receptor interaction. The low ratio of SDS to protein in the transferrin-receptor complex [14] may reflect preferential binding of SDS to membrane lipids with retention of protein-protein interactions as has been noted with Simliki virus [24]. Furthermore, the interactions of glycoproteins may be highly resistant to SDS dissociation because of strong associations of hydrophobic segments [25]. Results using sulfobetaine detergent with alkyl chain lengths of 8, 10, 12, or 14 carbon atoms indicate that dissociation of transferrin from its receptor is greatest with the longest chain length (unpublished results). The longer alkyl chains presumably reach deeper into the membrane and are thus more effective in disrupting hydrophobic interactions.

Clearly the transferrin-receptor complex, whether representing the bulk of bound transferrin as with SDS solubilization or only 30% as with the sulfobetaine 3-14, could be immunoprecipitated with antitransferrin antibodies. The most plausible explanation for co-immunoprecipitation of transferrin with a membrane protein is that the ^{125}I-labeled protein forms part, if not all, of the transferrin receptor. We obtained on occasion certain batches of goat antisera which although they effectively precipitated transferrin failed to precipitate transferrin when bound to its receptor, suggesting that the receptor was masking an antigenic site on the transferrin.

The reported molecular weights for the transferrin receptor vary widely from 60 000—190 000. A number of authors have previously used detergent solubilization and SDS-polyacrylamide gel electrophoresis to identify the putative

transferrin receptor. In general, these reports have used Triton X-100 or the closely related detergent Teric. The subsequent polyacrylamide gel electrophoretograms of the transferrin-receptor complexes isolated by gel filtration fraction is similar to the pattern shown in Fig. 3e, in that two moieties are often detected [7,9,11,13]. For example, Sullivan and Weintraub [11], who also used antitransferrin immunoprecipitation to increase specificity of receptor isolation and reported a pattern quite similar to the one obtained in this paper (Fig. 3e), tacitly assumed, as have other workers, that the protein migrating with the apparent molecular weight of transferrin was only transferrin. In our studies, depletion of reticulocytes of bound transferrin prior to iodination of the membrane allows us to state that the 80 000 molecular weight moiety is not transferrin but is a transferrin receptor protein. Although we did detect some radioactivity in the high molecular weight region of the polyacrylamide gel electrophoretograms, no clear peak of radioactivity could be detected compared to the control membranes. Hence, as suggested earlier [7,8,24], the higher molecular weight complex detected by gel filtration is presumably composed of two or three receptor subunits.

The evidence presented here indicates that proteins of apparent molecular weight 80 000 and 90 000 are part of the transferrin receptor when the receptor is analyzed by the Fairbanks system. When the same sample is analyzed using a gradient polyacrylamide system, only one band, with an apparent molecular weight of 80 000, is observed. These results imply that the two apparently different entities may represent modification of the same polypeptide chain, varying perhaps in the degree of glycosylation. A higher degree of glycosylation is known to decrease the charge/mass ratio and thereby alter the electrophoretic mobility in SDS-polyacrylamide gel electrophoresis [15].

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