Covalent Binding of Fatty Acid to the Transferrin Receptor in Cultured Human Cells*

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The human transferrin receptor could be fluorographically detected after immunoprecipitation from a leukemic T-cell line labeled with [3H]palmitic acid. The label was found only in association with the human transferrin receptor and not in association with two other major plasma membrane glycoproteins, demonstrating that the incorporation of radioactivity was not due to metabolism of the palmitate. Treatment of such complexes with dodecylsulfate-polyacrylamide-polsaturation of the [3H]palmitate-labeled transferrin receptor with hydroxylamine, prior to fluorography, resulted in release of a substantial fraction of the label from the molecule. In addition, at least part of the label released from immunoprecipitates of the transferrin receptor by treatment with hydroxylamine was identified as palmitoylthiohydantoin, providing further evidence that the labeled fatty acid is covalently bound to the receptor.

A proteolytic fragment (Mr = 70,000) derived from the portion of the transferrin receptor exposed on the cell surface can be obtained by trypsin digestion of intact or Nonidet P-40-solubilized cells. When cells were labeled with [3H]palmitic acid, none of the radioactivity could be detected in the tryptic fragment. Thus, the bound palmitate appears to be associated with the region of the molecule that is in close proximity to the plasma membrane.

Covalent attachment of fatty acids to proteins has been demonstrated in only a few cases. In bacteria, for example, Braun's lipoprotein of Escherichia coli cell wall (1) and references therein) is a case that has been extensively studied. In the case of animal viruses, Schmidt et al. have shown vesicular stomatitis virus G glycoprotein (2) and Sindbis virus E1 and E2 glycoproteins (3) to have covalently attached palmitate. Proteolipids isolated from the sarcoplasmic reticulum of rabbit skeletal muscle (4) or from brain white matter (5) were also found to have fatty acids covalently bound to protein moieties. More recently, Schlesinger et al. (6) presented evidence for fatty acid acylation of proteins from cultured human cell line. Their results suggest that covalent linkage of fatty acids to mammalian proteins may not be uncommon. However, none of the labeled proteins in these studies have been identified.

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Using a monoclonal antibody, we have identified a human cell surface antigen that is selectively expressed on proliferating cells (7, 8). This antigen is a glycoprotein with an apparent molecular weight of 95,000 under reducing conditions on SDS-polyacrylamide gels. Under nonreducing conditions, the apparent molecular weight is approximately 190,000 suggesting that the molecule exists in the membrane as a disulfide-bonded dimer. More recently, we have shown that this glycoprotein is the human transferrin receptor (9). In this report we describe another feature of the glycoprotein: its covalent association with fatty acid.

MATERIALS AND METHODS

Cell Lines—Human leukemic T-cell lines CCRF-CEM and RPMI 8402 were used (10). Both cell lines were grown in RPMI 1640 medium supplemented with 10% horse serum.

Radio-labeling Procedures—RPMI 8402 or CCRF-CEM cells (4 × 10⁶ cells/ml) were labeled with 100-125 μCi/ml of [9, 10-3H]palmitic acid (New England Nuclear, 17.6 Ci/mmol) for 4 h. The labeling medium was RPMI 1640 supplemented with 5% fetal calf serum dialyzed extensively against 0.15 M NaCl, 0.01 M phosphate buffer, pH 7.4. Metabolic labeling with [5-3H]methionine (Amersham, 1190 Ci/mmol) and cell surface lactoperoxidase-catalyzed iodination were carried out as previously described (11).

Immunoprecipitation and SDS-Polyacrylamide Gel Electrophoresis—Immunoprecipitates were prepared from 2 × 10⁶ cells using monoclonal antibodies that recognize, respectively, antigenic determinants on the human transferrin receptor, HLA, or the human homologue of murine T200 glycoprotein (7, 9, 12). Antibody-antigen complexes were collected with 0.15 ml of a 1% fixed Staphylococcus aureus suspension (v/v) and were then washed 3 times with 0.4 ml of 0.5% deoxycholate, 0.5% Nonidet P-40, 0.05% SDS in phosphate-buffered saline. The antigens were released in 40–70 μl of either reducing or nonreducing electrophoresis sample buffer (7, 13) by boiling for 2 min. After pelleting, 10–30 μl of each sample were subjected to SDS-polyacrylamide gel electrophoresis (7.5% or 10% gels) as previously described (11). After electrophoresis, gels were processed for fluorography (14) either directly or after fixing and staining. Both treatments gave similar results in terms of intensity of [3H]palmitate bands.

Hydroxylamine Treatment—The [3H]palmitate-labeled transferrin receptor was treated with hydroxylamine after SDS-polyacrylamide gel electrophoresis by dissolving the fixed gel in 50 ml of 1 M NH₄OH-HCl (Aldrich) titrated to pH 6.6 using NaOH. Incubation with hydroxylamine from 1 to 20 h gave similar results. Alternatively, to obtain sufficient material for analysis of the products formed, the transferrin receptor was treated with hydroxylamine after isolation by immunoprecipitation while still adsorbed to S. aureus. Eight immunoprecipitates were washed twice with 0.4 ml of cold phosphate-buffered saline to remove detergent, then mixed with 0.375 ml of 1 M NH₄OH-HCl (pH 9.8), and incubated at 37 °C for 90 min. After the fixed bacteria were removed by centrifugation, the supernatants (3 ml total volume) were extracted 3 times with 3 ml of CHCl₃/CH₃OH (2:1). The organic extracts were dried, redissolved in CHCl₃/CH₃OH (1:1) containing a mixture of pure palmitic acid and sodium palmitoylthiohydantoin, and then spotted on Silica Gel 60 plates (E. Merck) using toluene/methanol/acetic acid (90:20:1) for a solvent system as described in Ref. 6. R₄ values were determined after staining with I₂. Plates were then scraped and counted. Palmitoylthiohydantoin was synthesized according to Inoue and Yukawa (15) and detected as the ferric complex (16). It was kindly provided by Andreas Plückthun (Chemistry Dept., University of California, San Diego).

Tryptic Digest—Labeled cells (2 × 10⁶ cells/ml) were either directly treated with L-α-tosylamido-2-phenylethyl chloromethyl ketone-trypsin ( Worthington, 256 units/mg, 50 μg/ml for 20 min at 23 °C) or first dissolved at the same cell concentration in phosphate-

1 The abbreviation used is: SDS, sodium dodecyl sulfate.
buffered saline containing 1% Nonidet P-40 and then digested with 1-toyamido-2-phenethyl chloromethyl ketone-trypsin (250 μg/ml for 5 min at 23 °C) in a total volume of 150 μl. Proteolysis was terminated by adding either 100 μl of 5 mg/ml ovomucoid trypsin inhibitor or a mixture of 10 μl of 5 mg/ml ovomucoid trypsin inhibitor and 2 μl of 0.1 M phenylmethylsulfonyl fluoride in acetone.

RESULTS

Labeling of the Human Transferrin Receptor with [3H]-Palmitic Acid—Schlesinger et al. (6) showed that crude membrane preparations from human KB cells contain several proteins with covalently bound fatty acids, although the identity and cellular distribution of these proteins was not determined. These observations led us to test whether any previously identified plasma membrane glycoproteins of human lymphoid cells might contain covalently linked fatty acid. A human T leukemic cell line, CCRF-CEM, was incubated with [3H]palmitic acid for 4 h as described under “Materials and Methods.” Immunoprecipitates of three major membrane glycoproteins, the human transferrin receptor, the human homologue of murine T200 glycoprotein (11, 12), and HLA glycoprotein (17), were then prepared. As shown in Fig. 1, the transferrin receptor (M, = 95,000) was clearly labeled, whereas the other two glycoproteins were similar. Results were also obtained using another human T leukemic cell line, RPMI 8402 and, as expected, under nonreducing conditions the [3H]palmitate-labeled receptor migrated with an apparent molecular weight of about 190,000 (see Fig. 2b). Since in other experiments each of these molecules labeled to a similar extent with [35S]methionine, the specific labeling of the transferrin receptor with palmitate makes it unlikely that the incorporated label resulted from metabolic conversion of the palmitate to amino acids or carbohydrate. This conclusion is supported by data shown later that the radioactivity from the [3H]palmitate is associated with the region of the transferrin receptor proximal to the lipid bilayer.

Evidence That the Palmitate Label Is Covalently Associated with the Transferrin Receptor—Labeling of the transferrin receptor by palmitate seemed unlikely to be due to noncovalent interactions since immunoprecipitates were extensively washed in the presence of SDS, deoxycholate, and Nonidet P-40 (see “Materials and Methods”) and then boiled in electrophoresis sample buffer containing 2% SDS before electrophoresis.

However, to obtain additional evidence that the palmitate label was covalently associated with the transferrin receptor, SDS-polyacrylamide slab gels were treated with hydroxylamine prior to fluorography as described by Schlesinger et al. (6). Hydroxylamine is known to be nucleophilic to certain carboxylic acyl derivatives, in particular, esters, but not carboxylic acids, and upon reaction hydroxamic acids are formed (18). As shown in Fig. 2, a substantial loss of the label was observed when the transferrin receptor labeled with [3H]palmitic acid was exposed to hydroxylamine (compare tracks b and c with d and e). On the other hand, no detectable loss of radioactivity was observed when the [35S]methionine-labeled receptor was treated with hydroxylamine (compare tracks a and f). To identify the products formed upon reaction of the palmitate-labeled transferrin receptor with hydroxylamine, immunoprecipitates of the receptor were treated with hydroxylamine and then extracted with CHCl3:CH3OH (2:1), and the extracted radioactive species was analyzed using thin layer chromatography. Four radioactive species were resolved (Fig. 3) and could be visualized as discrete spots by autoradiography. One species migrated as palmitohydroxamate, the expected product of hydroxylamine substitution with a palmitic

Fig. 1. Fluorograph showing the labeling of the human transferrin receptor with [3H]palmitic acid. The figure shows a 10% polyacrylamide gel of immunoprecipitates isolated as described under “Materials and Methods” and dissolved in reducing electrophoresis sample buffer. The gel was stained with Coomassie blue, processed for fluorography, and exposed for 20 days. Immunoprecipitates were: transferrin receptor (Tr), HLA glycoprotein (HLA), and the human equivalent of murine T200 (Hu-T200).
Acid derivative. Palmitic acid was also identified and may result from hydrolysis of covalently bound palmitate under the alkaline conditions used as suggested by Schlesinger et al. (6) in earlier studies of palmitate-labeled proteins. Neither palmitic acid nor palmitohydroxamate were detected if immunoprecipitates were extracted and analyzed without treatment with hydroxylamine (data not shown). The two remaining radioactive species were not identified. Taken together, the data strongly suggest that the transferrin receptor is covalently associated with a fatty acid moiety.

The Region of the Transferrin Receptor Labeled with \[^3H\]Palmitate Is Proximal to the Lipid Bilayer—The region of the transferrin receptor that contained the palmitate label was identified in proteolysis experiments. Treatment of RPMI 8402 cells with as little as 2 μg/ml of trypsin resulted in the release, from the cell surface, of a soluble 70,000 molecular weight fragment of the transferrin receptor that was resistant to further proteolysis. As shown in Fig. 4a, the transferrin receptor is quantitatively cleaved by treatment of cells labeled by lactoperoxidase-catalyzed iodination with 50 μg/ml of trypsin at 23 °C for 20 min. The released fragment contained more than 90% of the radioactivity associated with the intact molecule and migrated with an apparent molecular weight of 70,000 under both reducing and nonreducing conditions (Fig. 4, a–d). Although proteolysis might take place at more than one site, a simple interpretation of these data would be that the bulk of the molecule is exposed on the external surface of the cell and that trypsin cleavage generates a large soluble fragment leaving the remainder of the molecule still containing a disulfide bond embedded in the membrane. Identical results were obtained when cell extracts, labeled with \[^{35S}\]methionine and dissolved in Nonidet P-40, were treated with trypsin (Fig. 4, e and f). In contrast, no radioactive species was obtained when lysates of cells labeled with \[^3H\]palmitate were treated with trypsin under identical conditions (Fig. 4i). Similar results were also obtained with intact CRF-CEM receptor with hydroxylamine as described under “Materials and Methods.”

**Fig. 3.** Analysis of products obtained from reaction of hydroxylamine with \[^3H\]palmitate-labeled transferrin receptor. The figure shows the results of thin layer chromatography of organic extracts obtained after treatment of \[^3H\]palmitate-labeled transferrin

**Fig. 4.** Tryptic digestion of the human transferrin receptor. Autoradiographs are shown of the transferrin receptor from RPMI 8402 cells radiolabeled with \(^{125}\text{I}\) using cell-surface lactoperoxidase-catalyzed iodination (a–d) or metabolically with either \[^{35S}\]methionine (e–g) or \[^3H\]palmitic acid (h–j). The figure shows immunoprecipitates prepared from intact cells treated with trypsin (a, c, f), cell extracts treated with trypsin (e and i), an inhibition control in which protease inhibitors were added to a sample of cell lysate before addition of trypsin (h), and samples not treated with trypsin (b, d, g, j). Also shown are conditions used for electrophoresis (reducing or nonreducing). Mr corresponds to the apparent molecular weight for tracks a–d only.
cells and lysates (data not shown). This strongly suggests that the [3H]palmitate label is exclusively associated with the region of the transferrin receptor proximal to the lipid bilayer.

**DISCUSSION**

In this report, we have presented evidence for the covalent association of palmitic acid with the cell surface transferrin receptor of cultured human T leukemic cell lines. Thus, we have extended earlier observations suggesting that certain proteins in cultured human cells contain fatty acid residues (6), by identifying a specific plasma membrane glycoprotein that is modified in this manner.

The functional significance of the covalent attachment of fatty acid to the transferrin receptor is not clear. One possibility is that the covalently bound fatty acid anchors the glycoprotein in the plasma membrane. This would be consistent with the observation that [3H]palmitate labels only that portion of the transferrin receptor containing the region that interacts with the lipid bilayer. It is noteworthy that neither of the transmembrane glycoproteins HLA (17) nor human T200 (11, 12 and Footnote 2) glycoproteins labeled with [3H]palmitate. Consequently, covalent fatty acid attachment may not be a general feature of transmembrane glycoproteins and may indicate that the transferrin receptor represents a different class of membrane-bound glycoproteins, particularly since we have not been able to clearly demonstrate that the receptor is exposed on the cytoplasmic side of the plasma membrane.

Another possible role for the addition of fatty acid residues to membrane glycoproteins is in their transport to the cell surface. Studies of the vesicular stomatitis virus G glycoprotein suggest that addition of fatty acid to this molecule may occur as the glycoprotein moves to the plasma membrane (19, 20). Covalent binding of fatty acid to the transferrin receptor offers a new model for studying fatty acid biosynthetic modification of glycoproteins.

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