Gene Transfection-mediated Overexpression of β1,4-N-Acetylglucosamine Bisecting Oligosaccharides in Gliona Cell Line U373 MG Inhibits Epidermal Growth Factor Receptor Function*

(Received for publication, December 17, 1996, and in revised form, February 10, 1997)

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N-linked oligosaccharides appear to be important for the function of the epidermal growth factor (EGF) receptor. In a previous study (Rebbaa, A., Yamamoto, H., Moskal, J. R., and Bremer, E. G. (1996) J. Neurochem. 67, 2265–2272), we showed that binding of the erythroagglutinating phytohemagglutinin lectin from Phaseolus vulgaris to the bisecting structures on the EGF receptor from U373 MG glioma cells blocked EGF binding and receptor autophosphorylation. In this study we examined the consequences of overexpression of the bisecting structure on the EGF receptor by gene transfection of U373 MG cells with the N-acetylgalcosaminyltransferase III (GnT-III). This modification leads to a significant decrease in EGF binding and EGF receptor autophosphorylation. In addition, the cellular response to EGF was found to be altered. Proliferation of U373 MG cells in serum-free medium is inhibited by EGF. In contrast, proliferation of the GnT-III-transfected cells was stimulated by EGF. These data demonstrate that changes in EGF receptor glycosylation by GnT-III transfection reduces the number of the active receptors in U373 MG cells and that this change results in change in the cellular response to EGF.

Glycosylation of the epidermal growth factor (EGF) receptor is essential for its function (1). Treatment of A431 cells that overexpress EGF receptor, with N-linked glycosylation inhibitors such as tunicamycin or glucosamine, reduced ligand binding by more than 50% (2). Addition of the oligosaccharide moiety to EGF receptor during its biosynthesis was found to be essential for the acquisition of EGF binding to the receptor (3). Binding of lectins to the EGF receptor also modulates the receptor function. Concanavalin A and wheat germ agglutinin lectin inhibit EGF binding to the receptor (4). More recently, concanavalin A was described to be a potent inhibitor of EGF receptor autophosphorylation and signaling, suggesting that

The abbreviations used are: EGF, epidermal growth factor; DMEM, Dulbecco’s modified Eagle’s medium; E-PHA, erythroagglutinating phytohemagglutinin lectin from P. vulgaris; FBS, fetal bovine serum; GnT-III, UDP-N-acetylgalcosamine β1,4-N-acetylgalcosaminyltransferase III; PBS, phosphate-buffered saline; kb, kilobase(s).

*This work was supported in part by grants from the Illinois division of the American Cancer Society (to H. Y.) and from the Mizutani Foundation for Glycoscience (to E. G. B.) and National Institute of Health Grant NS33383 (to E. G. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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This paper is available online at http://www.jbc.org/jbc/9275

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buffered saline (PBS), and the monolayer was solubilized by the addition of 200 μl of lysis buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 100 mM NaF, 1 mM MgCl₂, 1.5 mM EGTA, and 1% Nonidet P-40 (5). 50 μg of protein was applied to an 8% polyacrylamide gel, electro-transferred to an Immobilon P membrane (Millipore, Bedford, MA), and probed with biotinylated E-PHA (Vector Labs, Burlingame, CA). Reactive glycoproteins were detected by streptavidin-peroxidase and ECL reagents (Amersham Corp.) (5).

Analysis of the change in EGF receptor glycosylation was carried out by immunoprecipitation of the receptor; total cell lysates (500 μg) were incubated at 4 °C overnight with 5 μg of anti-EGF receptor monoclonal antibody (clone F4, Sigma) complexed with rabbit anti-mouse IgG and protein A-agarose (Sigma). After washing the pellet, 30 μl of Laemmlí buffer (2×) was added for 5 min, and the supernatant was separated by SDS-polyacrylamide gel electrophoresis. The presence of the bisecting oligosaccharide structure on the precipitated EGF receptor was detected by incubation with biotinylated E-PHA. Reactive glycoproteins were detected by streptavidin-peroxidase and ECL reagents.

Detection of EGF Receptor Protein at the Cell Surface—Cells were seeded at 10⁵ cell/well in 24-well plates and incubated overnight at 37 °C in DMEM containing 10% FBS. The cells were then washed three times with PBS and fixed with 10% buffered formalin for 20 min at room temperature. After three additional PBS washes, the fixed cells were incubated for 1 h at room temperature in PBS containing 10 μg/ml anti-human EGF receptor antibody (clone LA1; Upstate Biotechnology, Lake Placid, NY). The cells were then washed with PBS and incubated for 30 min at room temperature with 1 μg/ml of 125I-labeled anti-mouse IgG (DuPont NEN) followed by three PBS washes. Nonspecific binding was determined by the addition of radiolabeled anti-mouse antibody to cells without anti-EGF receptor antibody. Cells were solubilized in 200 μl of 0.2 N NaOH, and radioactivity was detected with a liquid scintillation counter.

EGF Binding Assays—For cell surface binding, the cells were seeded at a density of 5 × 10⁴ cells/ml in 24-well plates and incubated overnight in DMEM containing 10% FBS. The medium was then replaced with phenol red-free DMEM, after 15 of incubation at 37 °C, the cells were washed twice with binding buffer (PBS containing 0.1% bovine serum albumin). 125I-EGF (10⁵ cpm) was then added in the presence of unlabeled EGF over a concentration range of 0–100 nM. Nonspecific binding was determined by the addition of 1 μM cold EGF. After incubation for 1 h at room temperature, unbound EGF was aspirated, and the cells were washed three times with binding buffer. The cells were removed by the addition of 200 μl of 0.2 N NaOH to each well. Bound radioactivity was then counted in a γ counter (8). The KD and the total number of EGF binding sites were determined from the specific binding counts by the method of Scatchard (9).

For binding to the immunoprecipitated receptor, EGF receptor immunoprecipitated on protein A-agarose beads were then incubated with 50,000 cpm 125I-EGF and 10 nM cold EGF for 15 min on ice. Nonspecific binding was measured by the addition of 1 μM cold EGF. After incubation for 1 h at room temperature, unbound EGF was aspirated, and the cells were washed three times with binding buffer. The cells were then centrifuged (12,000 × g for 1 min) and washed four times with cold PBS. Radioactivity associated with the immunocomplex-agarose pellet was measured in a γ counter.

EGF Receptor Autophosphorylation—Cells were incubated in serum-free medium supplemented with 0–100 ng/ml of EGF for 10 min or with 100 ng/ml of EGF for 1–15 min at 37 °C. After washing twice with ice-cold PBS, the monolayer was solubilized with 200 μl of lysis buffer (5). 50 μg of soluble protein was resolved by SDS-polyacrylamide gel electrophoresis, electro-transferred onto an Immobilon P membrane (Millipore, Bedford, MA), and probed with biotinylated E-PHA (Vector Labs, Burlingame, CA). Reactive glycoproteins were detected by incubating the membrane for 1 h at room temperature with a mouse monoclonal anti-phosphotyrosine antibody (PY-20; Transduction Laboratories, Lexington, KY). Complexes were detected by sequential blotting with biotinylated goat anti-mouse IgG linked to peroxidase. Phosphorylated proteins were detected with ECL reagents (5).

1H-Thymidine Incorporation—Cells were seeded in 24-well plates at 5 × 10⁴ cells/ml in 10% FBS containing DMEM medium and incubated at 37 °C for 24 h. The medium was then replaced by DMEM without FBS, and the cells were incubated with different concentrations of EGF for another 24 h at 37 °C. 1H-Thymidine (1 μCi/ml) was then added to each well and incubated for 5 h at 37 °C (10). The cells were then washed three times with ice-cold PBS, incubated with 5% trichloroacetic acid at 4 °C for 30 min, followed by three washes with ice-cold trichloroacetic acid. The trichloroacetic acid-precipitable radioactivity was solubilized with base and counted in a liquid scintillation counter.

RESULTS AND DISCUSSION

The role of the oligosaccharide portion in the modulation of the EGF receptor function has been mainly examined by inducing transient modifications in receptor glycosylation, by incubation of cells with glycosylation inhibitors (2) or lectins (4, 11). Through these approaches, the function of EGF receptor was abolished in cells where glycosyltransferases were inhibited as well as when the cells were treated with certain lectins. Glycosylation of the EGF receptor seems to be tissue-specific. In human carcinoma cell line A431 for example, EGF receptor was found to contain mainly high mannos oligosaccharide structure (2).

In a human glioma cell line, U373 MG, EGF receptors contain the bisecting oligosaccharide structure, and interaction of this structure with E-PHA lectin was found to inhibit ligand recognition by the receptor (5).

Stable GnT-III transfectants were generated in a glioma cell line, U373 MG, to determine the consequences of overexpression of the bisecting oligosaccharide structure on EGF receptor binding and function because E-PHA had such a profound effect on binding and function (5). Transfection was verified by GnT-III mRNA expression, which was very high in transfected cells compared with parental cells (Fig. 1A). A prominent band at approximately 2.2 kb was expressed only in the GnT-III-transfected cells (Lane 2). This band corresponds with the expected message size for the transfected GnT-III. A faint band was also detected at 4.7 kb and is consistent with the endogenous GnT-III (6).

Overexpression of GnT-III mRNA in transfected cells should result in an increase in bisecting GlcNAc products. The presence of GnT-III enzyme products was determined by measuring the increase in E-PHA lectin binding to total proteins extracted from transfected and nontransfected cells. As shown in Fig. 1C, a significant increase in E-PHA binding to the GnT-III-transfected cells was observed when compared with the parental cells or those transfected only with vector alone (pcDNA3). Most of the glycoproteins affected were in the molecular mass range of 70–120 kDa. These data are in accordance with the findings of Miyoshi et al. (12) that GnT-III transfection of HB511 cells altered N-linked branching of proteins in a similar molecular mass range. The identity of these proteins has not yet been determined. A 67-kDa protein, however, was reported by Ross and collaborators (13) to be an excellent substrate for GnT-III upon treatment of LAN-5 human neuroblastoma cells with retinoic acid.

Glycosylation of the EGF receptor was analyzed on immunoprecipitated receptor (Fig. 1D). In lanes 1 and 2, the blot was probed with biotinylated E-PHA lectin. The 170-kDa glycoprotein, which corresponds to EGF receptor, reacted with the E-PHA lectin from both cell types by anti-EGF receptor antibody. Reactivity of EGF receptor appeared to be equivalent in both cell types. Taken together, these data indicate that GnT-III transfection was able to increase the amount of bisecting oligosaccharide structure on EGF receptor expressed in the transfected U373 MG cells.

The binding of EGF to its cell surface receptor on U373 MG parental and GnT-III-transfected U373 MG cells was examined. The GnT-III-transfected cells were found to bind much less EGF than the nontransfected cells (Fig. 2). The apparent number of EGF receptors at the cell surface was reduced from about 7 × 10⁵ in the parental cells to about 3 × 10⁵ in the GnT-III-transfected cells (Table I). The binding affinity, however, did not seem to be affected.
The decrease in EGF binding at the cell surface may be explained by a decrease in the number of active receptors on the cell surface. This may be due to a decrease in receptor biosynthesis or to transport to the cell membrane or as a consequence of the change in EGF receptor glycosylation. The expression of EGF receptor protein at the cell surface was determined by incubating the cells with monoclonal anti-EGF receptor antibody and 125I-labeled goat anti-mouse IgG. As shown in Table I, parental and GnT-III-transfected cells bound equivalent quantities of anti-EGF receptor antibody. These data suggest that similar quantities of EGF receptor protein are expressed on the cell surface of these two cell types. 125I-EGF binding to equal amounts of immunocomplexed receptors from transfected and nontransfected cells was also determined (Fig. 3). Similar to the reduction observed in cell surface binding, EGF binding to the immunoprecipitated receptor was also reduced by about 50%. These data and those shown in Fig. 1D suggested that the biosynthesis or transport to the cell surface of EGF receptors was not affected by GnT-III transfection of U373 MG cells. Furthermore, the overexpression of bisecting oligosaccharide structures on EGF receptor appears to be responsible for the reduction of EGF binding because the inhibition was still observed on equal amounts of immunoprecipitated EGF receptor protein. A possibility suggested by these results is that there are two populations of receptors: one that binds EGF in a manner that is similar to that of the wild type receptor and a second that is incapable of binding EGF. The alteration in EGF receptor glycosylation by GnT-III transfection may be responsible for the inhibition of EGF binding to these receptors. One or more N-linked oligosaccharide chains on or near the EGF binding site may be responsible for the reduction of EGF binding.
phosphorylation in the GnT-III-transfected cells compared with the parental cells. The diminution in EGF receptor activation in transfected cells agrees with our finding on EGF binding and suggests that the modulation of the oligosaccharide branching by GnT-III on the EGF receptor may disturb the activity and subsequently the signaling cascade initiated by this receptor.

One of the final outcomes of EGF signaling is cell proliferation. Addition of EGF to cultures of many tumor cell lines that overexpress the EGF receptor, however, is inhibitory for cell growth (14–16). The human epidermoid carcinoma cell line A431 is an example of a line that overexpresses EGF receptor and is growth inhibited by EGF (14). A431 cells can be subcloned into two populations that respond differently to EGF. The A1S subclone proliferates in the presence of EGF, and the A5I subclone, like the parental A431, is growth inhibited by EGF (17, 18). One of the major differences between these clones is the number of EGF receptors. A1S cells contain about one-half the number of receptors as A5I (17). This type of data has suggested a possible inverse relationship between EGF receptor number on the cell surface and EGF-induced mitogenesis.

Human gliomas and glioma cell lines also overexpress EGF receptor (19–21). In our study, U373 MG cells transfected with GnT-III gene were found to grow 50% slower than the parental cells (data not shown). The proliferative response to EGF, however, was found to be different between U373 MG cells and the GnT-III-transfected cells. U373 MG cells are growth inhibited by EGF. As shown in Fig. 5, addition of EGF to the cell culture medium had an inhibitory effect on thymidine incorporation into U373 MG cells. There was about 40% inhibition with less than 1 ng/ml of EGF. There was still at least 50% when 100 ng/ml of EGF was added to the medium (data not shown). The GnT-III-transfected cells, on the other hand, were stimulated in the presence of low concentrations of EGF (from 1 to about 5 ng/ml). This response was dose-dependent and was maximal at

FIG. 3. Binding of 125I-EGF to the immunoprecipitated EGF receptor. EGF receptor was immunoprecipitated from 500 μg of protein from total cell lysate. The immunocomplex was incubated with 105 cpm radiolabeled EGF and 10 nM cold EGF for 15 min on ice. Unbound EGF was removed by washing the pellet four times with cold PBS, and the radioactivity associated to the pellet was measured. Data are averages ± S.E. (bars) of triplicate determinations. The inset in this figure represents a Western blot showing the expression of EGF receptor in U373 MG and GnT-III-transfected cells. The blot was stained with anti-EGF receptor monoclonal antibody F4. The immunoreactive complex was then detected by peroxidase and ECL reagents.

FIG. 4. EGF-dependent protein tyrosine phosphorylation in U373 MG parental and transfected cells. Cells were untreated or treated with the indicated concentrations of EGF for 10 min (A) or with 100 ng/ml of EGF for the indicated times (B). The proteins (50 μg) from these cells were electrophoresed and transferred to an Immobilon P membrane. Phosphorylated proteins were detected with anti-phosphotyrosine antibody followed by peroxidase and ECL reagents as described under “Experimental Procedures.”

FIG. 5. Proliferative response to EGF. Parental and GnT-III-transfected U373 MG cells were seeded in 24-well plates in 10% FBS containing DMEM medium. After 24 h at 37 °C, the medium was replaced with serum-free medium, and EGF was added at the indicated concentrations. Thymidine incorporation was measured as indicated under “Experimental Procedures.” Data are averages ± S.E. (bars) of triplicate determinations. Where no error bar is present, the S.E. was smaller than the symbol.
about 2 ng/ml EGF. This might be explained by the overexpression of the bisecting structure on EGF receptor reducing the apparent number of EGF binding sites on the cell surface. Similar to the epidermoid carcinoma lines described above, the EGF-dependent proliferation of these cells is inversely proportional to the number of binding sites on the cell surface. As the number of EGF binding sites decrease on these tumor cell lines there is a change from inhibition to stimulation of proliferation by EGF.

In summary, this study demonstrates that overexpression of the GnT-III products on EGF receptor in U373 MG cells induces significant changes in EGF binding and receptor autophosphorylation. The proliferative response of the cells to EGF was also changed after transfection. The mechanism by which overexpression of bisecting structure in glioma cells alters their growth behavior remains to be determined.

Acknowledgments—We thank Dr. Barbara Mania-Farnell for help in the preparation of this manuscript.

REFERENCES
1. Soderquist, A. M., Todderud, G., and Carpenter, G. (1988) Adv. Exp. Med. Biol. 231, 569–582
2. Soderquist, A. M., and Carpenter, G. (1984) J. Biol. Chem. 259, 12586–12594
3. Slieker, L. J., and Lane, M. D. (1985) J. Biol. Chem. 260, 687–690
4. Zeng, F. Y., Benguria, A., Kafert, S., Andre, S., Gabius H. J., and Villalobo, A. (1995) Mol. Cell. Biochem. 142, 117–124
5. Rebbaa, A., Yamamoto, H., Maskai, J. R., and Bremer, E. G. (1996) J. Neurochem. 67, 2265–2272
6. Ibara, Y., Nishikawa, A., Tohma, T., Soejima, H., Niikawa, N., and Taniguchi, N. (1993) J. Biol. Chem. 113, 692–698
7. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
8. Miyaji, K., Tani, E., Shindo, H., Nakano, A., and Tokunaga, T. (1994) J. Neurosurg. 81, 411–419
9. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660–665
10. Wakebull, E. M., and Wharton, W. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 8513–8517
11. Feizi, T., and Childs, R. A. (1985) Trends Biochem Sci. 10, 24–29
12. Miyoishi, E., Nishikawa, A., Ibara, Y., Saiko, H., Oozumi, N., Hayashi, N., Funamoto, H., Kamada, T., and Taniguchi, N. (1985) J. Biol. Chem. 270, 6216–6220
13. Ross, A. A., Jones, C. S., and De Luca, L. M. (1995) Int. J. Cancer. 62, 303–308
14. Kawamoto, T., Mendelsohn, J., Le, A., Sato, G. H., Lazar, C. S., and Gill, G. N. (1984) J. Biol. Chem. 259, 7761–7766
15. Ennis, B. W., Valverius, E. M., Bates S. E., Lippman, M. E., Belot, F., Kris, R., Schlessinger, J., Masui, H., Goldenberg, A., Mendelsohn, J., and Dickson, R. B. (1989) Mol. Endocrinol. 3, 1830–1838
16. Armstrong, D. K., Kaufmann, S. H., Ottaviano, Y. L., Furuya, Y., Buckley, J. A., Isaacs, J. T., and Davidson, N. E. (1994) Cancer Res. 54, 5280–5283
17. Barns, D. (1987) Methods Enzymol. 146, 88–92
18. Buss, J. E., Kudlow, J. E., Lazar, C. S., and Gill, J. N. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2574–2581
19. Olson, J. J., James C. D., Krishk, A., Barnett, D., and Hunter, S. (1995) Neurosurgery 36, 740–748
20. Hunter, S., Abbitt, K., Varma, V. A., Olson, J. J., Barnett, D. W., and James, C. B. (1995) J. Neuropathol. Exp. Neurol. 54, 57–64
21. Sauter, G., Maeda, T., Waldman, F. M., Davis, R. L., and Feuerstein, B. G. (1996) Am. J. Pathol. 148, 1047–1053
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J. Biol. Chem. 1997, 272:9275-9279.
doi: 10.1074/jbc.272.14.9275

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