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The *her*-2/*neu* oncogene stimulates the transcription of *N*-acetylglucosaminyltransferase V and expression of its cell surface oligosaccharide products

Lin Chen[1], Weijie Zhang[2], Nevis Fregien[3] and Michael Pierce[1]

[1]*Department of Biochemistry and Molecular Biology and Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia 30602, USA;* [2]*Molecular Biology Institute, Department of Biochemistry and Molecular Biology, Shanghai Jaio-Tung University, Shanghai, China;* [3]*Department of Cell Biology and Anatomy, University of Miami Medical School, Miami, Florida 33101, USA*

Malignant transformation is associated with changes in the glycosylation of cell surface proteins. For example, the N-linked oligosaccharides containing the [GlcNAcβ(1,6)Man] branch are increased after transformation of many cell types by a number of tumor viruses and oncogenes which induce the expression of N-acetylglucosaminyl-transferase V (GlcNAc-T V), the enzyme that adds this branch. A large percentage of human breast carcinomas have increased N-linked β(1,6) branches on glycoproteins, while up to 30% of breast carcinomas have amplified the oncogene *her*-2/*neu* (*erb*-B2). We tested the hypothesis that expression of *her*-2/*neu* stimulates GlcNAc-T V gene expression and increases the β(1,6) branching of N-linked oligosaccharides. We found that *neu*-transformed NIH3T3 cells have a threefold increase in GlcNAc-T V enzyme activity and increased β(1,6) branching on a specific set of glycoproteins. Promoter/reporter experiments showed that *her*-2/*neu* stimulates transcription from the human GlcNAc-T V promoter and that the *her*-2/*neu* response element was located about 400 bp 5′ of the transcription initiation site and includes three Ets transcription factor binding sequences. Co-transfections with dominant-negative Raf and Ets expression plasmids demonstrated that the transcriptional activation of the GlcNAc-T V promoter by *neu* is mediated by the Ras-Raf-Ets signal transduction pathway.

Keywords: *neu*/*her*-2; oncogene; glycosyltransferase; glycosylation

Introduction

Oncogenic transformation of many cultured cell types causes changes in types and amounts of oligosaccharide expression. A common alteration involves the increase in particular N-linked oligosaccharides when cells are transformed by a number of tumor viruses, including Rous sarcoma virus and polyoma virus, and oncogenes such as *ras* and *fps*/*yes* (Buck *et al*., 1971; Dennis *et al*., 1989; Le Marer *et al*., 1992; Lu and Chaney, 1993; Pierce and Arango, 1986; Yamashita *et al*., 1984). These oligosaccharides contain a β(1,6) branched N-acetylglucosamine (GlcNAc) and their synthesis is catalyzed by the glycosyltransferase, N-acetylglucosaminyltransferase V (E.C.2.4.1.155, GlcNAc-T V) (Arango and Pierce, 1988). This enzyme is localized in the Golgi apparatus, and transfers a GlcNAc from UDP-GlcNAc to the α(1,6)-linked mannose of Asn-linked biantennary and triantennary oligosaccharides in a β(1,6) linkage (Cummings *et al*., 1982), shown by the following scheme:

$$\text{UDP} - \mathbf{GlcNAc} + \text{GlcNAc}\beta(1,2)\text{Man}\alpha(1,6)\text{Man} - \text{R}$$

$$\begin{array}{c} \text{GlcNAc} \\ \vert \\ \beta(1,6) \end{array}$$

$$\longrightarrow \text{GlcNAc}\beta(1,2)\text{Man}\alpha(1,6)\text{Man} - \text{R} + \text{UDP}$$

$$\text{GlcNAc} - \text{TV}$$

The β(1,6) branched products of the GlcNAc-T V reaction are often further modified by the addition of additonal sugars to form polylactosamines. These β(1,6) branched oligosaccharides are expressed on a variable but select set of secreted and cell surface glycoproteins (Do *et al*., 1994; van den Eijnden *et al*., 1988), including several adhesion molecules (Arango and Pierce, 1988; Demetriou *et al*., 1995; Kawano *et al*., 1993). Direct transformation of Mv1Lu mink lung cells with a cDNA encoding the rat GlcNAc-T V indicated that over-expression of GlcNAc-T V can alter cell adhesion and migration properties (Demetriou *et al*., 1995). Other studies have used L-phytohemagglutinin (L-PHA), which is toxic to cells to which it binds (Stanley, 1983), to select for variants of metastatic cells with reduced levels of GlcNAc-T V activity and β(1,6) branching. These variants have reduced metastatic potential (Dennis *et al*., 1987; Lu *et al*., 1994).

Several studies have examined the over-expression of β(1,6) branched N-linked oligosaccharides in human tumors, in particular human breast carcinomas (Dennis and Laferte, 1989; Fernandes *et al*., 1991). The level of L-PHA staining level was shown to correlate with disease progression from fibroadenoma through hyperplasia with atypia to carcinoma (Fernandes *et al*., 1991). In non-diseased adult breast epithelial cells there is little or no detectable L-PHA staining (Li and Roth, 1997).

Recent experiments have shown that src tyrosine kinase activity induces the expression of the GlcNAc-T V gene at the level of transcription (Buckhaults *et al*., 1997). In addition, up to 30% of human breast

Correspondence: M Pierce
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carcinomas have elevated tyrosine kinase activity due to the amplification and over-expression of the oncogene, *her*-2 (Kraus *et al*., 1987; Slamon *et al*., 1987, 1989; Van de Vijver *et al*., 1987). We hypothesized, therefore, that GlcNAc-T V expression might also be up-regulated by the *her*-2 oncogene, or its mouse homolog, *neu*, as previously shown for *src*. We show here that expression of this oncogene in NIH3T3 fibroblasts up-regulates GlcNAc-T V activity, which results in increased β(1,6) branching of a set of glycoproteins. This mechanism of stimulation operates at the transcriptional level and involves the signal transduction pathway that includes Ras, Raf and the Ets family of transcription factors.

Results

Increased GlcNAc-T V activity and β(1,6) branching in neu-*transfected NIH3T3 cells*

The level of expression of cell surface N-linked oligosaccharides that contain β(1,6) branches is increased after transfection by several oncogenes, including *src*, which encodes for a tyrosine kinase (Dennis *et al*., 1989; Pierce and Arango, 1986; Yamashita *et al*., 1984). Furthermore, this increase in β(1,6) branched oligosaccharides is the result of the specific induction of the expression of N-acetylglucosaminyltransferase V (GlcNAc-T V) which catalyzes the addition of this branch (Arango and Pierce, 1988b; Yamashita *et al*., 1985). Another tyrosine kinase oncogene, *her*-2/*neu* which is often over-expressed in breast carcinomas, was reported to cause altered morphology, increased invasiveness and higher metastatic potential (Yu and Hung, 1991), suggesting that this oncogene might also affect the amounts of cell surface β(1,6) branched oligosaccharides on transfected cells. To test the hypothesis, the amounts of β(1,6) branched oligosaccharides present on NIH3T3 cells and *neu*-transfected NIH3T3 cells (Yu and Hung, 1991) were compared. The relative amounts of β(1,6) branched oligosaccharides were determined by staining these cells with FITC-labeled L-PHA, which binds specifically to these oligosaccharides, and examining the binding using confocal fluorescence microscopy. The fluoromicrographs shown in Figure 1 revealed that *neu*-transfected NIH3T3 cells showed significantly more L-PHA binding than the untransfected NIH3T3 cells, indicating that *neu* transfection, like *src*, caused an increase in the amount of the β(1,6) branch to the N-linked oligosaccharides.

In order to ascertain if the increased β(1,6) branching was caused by an increase in the levels of GlcNAc-T V in the *neu*-transfected NIH3T3 cells, the specific activities of two glycosyltransferases involved in N-linked oligosaccharide synthesis, GlcNAc-T V and N-acetylglucosaminyltransferase I (GlcNAc-T I), were compared in NIH3T3 and *neu*-transfected NIH3T3 cells. Indeed, the *neu*-transfected NIH3T3 cells displayed approximately three times more GlcNAc-T V activity as the parental cells, while GlcNAc-T I showed no change in specific activity (Figure 2). These data demonstrate that the constitutively activated *neu* construct induced the expression of GlcNAc-T V, but this effect was not observed for another N-acetylglucosaminyltransferase that functions in the biosynthesis of N-linked complex oligosaccharides.

An RNA gel blot analysis was performed to determine whether there was an increase in the

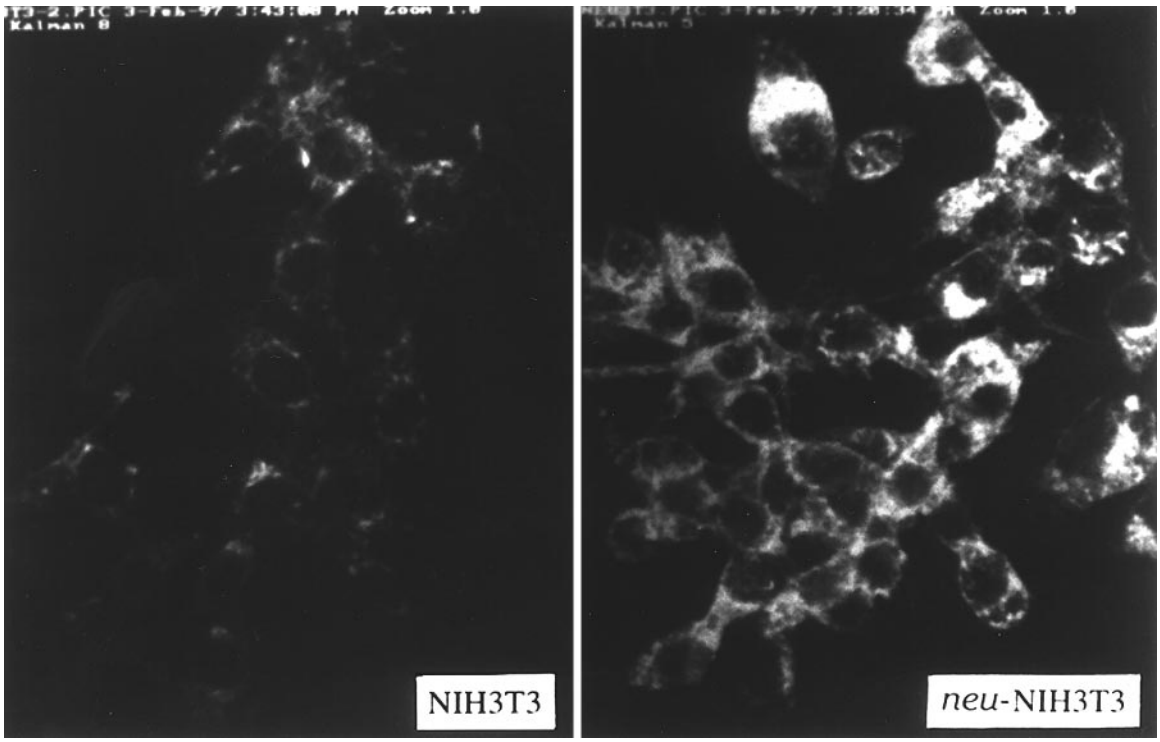

Figure 1 Staining of 3T3 and *neu*-3T3 cells with FITC-conjugated L-PHA. Cells were grown on chamber slides and permeabilized prior to incubation with FITC-L-PHA (50 μg/ml). Detection was with a confocal fluorescence microscope. Left panel, NIH3T3 cells; right panel, *neu*-transfected NIH3T3 cells

amount of GlcNAc-T V mRNA in the *neu*-expressing cells. Hybridization with a GlcNAc-T V cDNA probe to equivalent amounts of total RNA prepared from NIH3T3 and *neu*-transfected NIH3T3 cells showed threefold more GlcNAc-T V message in the *neu*-transfected cells (Figure 3). These results show that *neu* tyrosine kinase activity affects the level of GlcNAc-T V mRNA accumulation and possibly the rate of GlcNAc-T V gene transcription.

GlcNAc-T V induction by her2/neu *is mediated by the ras-raf-ets-2 signalling pathway*

Previous studies have shown that *src* tyrosine kinase activity could stimulate transcription from the GlcNAc-T V promoter (Buckhaults *et al*., 1997). To determine whether *neu* also affects the transcription from the GlcNAc-T V promoter, transfection experiments were performed using previously constructed GlcNAc-T V promoter/luciferase reporter plasmids (depicted in Figure 4) with and without a *neu* expression plasmid. As shown in Figure 5, upper panel, pGL2b-TV1, pGL2b-TV2 and pGL2b-TV3 constructs showed significant induction of luciferase activity when co-transfected with the *neu* expression plasmid into reporter HepG2 cells. However, pGL2b-TV4, which contained the smallest promoter fragment, showed little response to *neu* expression. These results indicate that neu tyrosine kinase activity does stimulate transcription from the GlcNAc-T V promoter, and that a *neu* response element is located between 659 and 269 bases 5′ of the transcription initiation site.

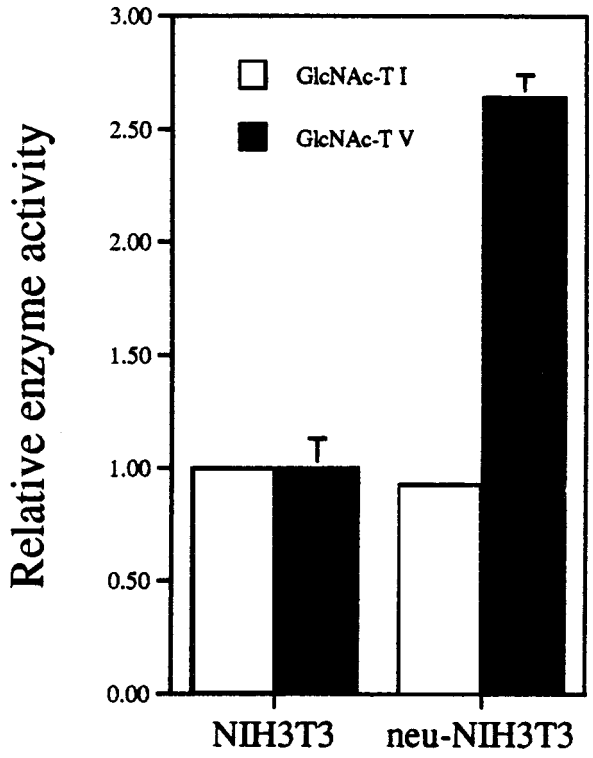

Figure 2 GlcNAc-T V and GlcNAc-T I specific activities in 3T3 and *neu*-3T3 cells. The specific activities of both enzymes were determined from cell lysates at three protein concentrations and in triplicate. The results are plotted normalized to the specific activity of the 3T3 cells

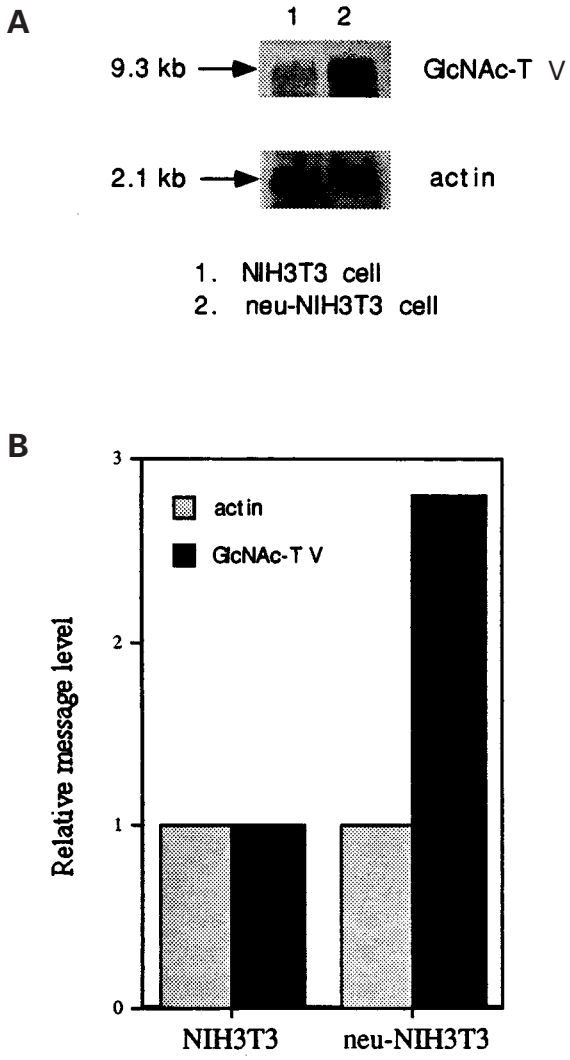

Figure 3 Messenger RNA levels of GlcNAc-T V in 3T3 and *neu*-3T3 cells. (**a**) Northern analysis of RNA from 3T3 and *neu*-3T3 cells probed with 1 kb fragment of rat GlcNAc-T V cDNA (upper panel); a human actin cDNA, as a loading control (lower panel). (**b**) GlcNAc-T V and actin mRNA band intensities shown in **a** were quantified with a phosphorimager, and the results were plotted normalized to the levels of each mRNA in the 3T3 cells

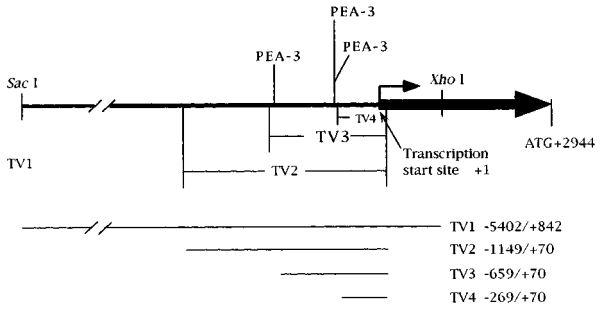

Figure 4 The 5′ region of the human GlcNAc-T V gene. TV1 is a *Sac*I/*Xho*I genomic fragment from the human GlcNAc-T V gene and includes 848 bp of the 5′-untranslated region. TV2, TV3 and TV4 were generated by PCR techniques as described in Buckhaults *et al*. (1997). The TV3 fragment contains the shortest sequence tested that responded to *neu* activation and includes three PEA-3 sites which are bound by Ets-2)

To elucidate further the pathway of signal transduction between *neu* and the GlcNAc-T V promoter, additional co-transfections were performed with the promoter reporter plasmids and expression plasmids encoding potential signal transduction molecules. Analysis of the DNA sequence in the GlcNAc-T V promoter between bases −659 and −269 revealed the presence of several consensus Pea-3 sites. These sites have been shown to be the binding sites for the transcription factor Ets-2, which in some cases is a downstream effector of the *neu* signal transduction pathway (Galang *et al*., 1996). In addition, it has been shown that *ras*-mediated induction of expression from Ets-driven promoters requires Raf-1 kinase (Bruder *et al*., 1992; Buttice and Kurkinen, 1993), and dominant-negative expression plasmids for both Ets-2 and Raf-1 were available. Therefore, the role of these molecules in the induction of GlcNAc-T V promoter activity by *neu* was investigated. Figure 5, middle panel, shows the effects on transcription from the GlcNAc-T V promoter of co-transfecting HepG2 cells with an expression plasmid for *neu*, with and without a plasmid encoding the dominant-negative Ets-2 (Δ1–328ets-2). The co-expression of the Δ1–328ets-2 plasmid blocks the *neu* induction of transcription, indicating that ets is required for the *neu* effect.

In similar experiments, the pGL2-TV-3 reporter plasmid was co-transfected with *neu* with and without a mutant *raf* expression plasmid encoding RafC4B, which has a defective kinase. This mutant blocks the *ras* signaling pathway by binding to ras, but cannot transmit the signal due to its defective kinase. As shown in Figure 5, lower panel, the expression of rafC4B in the transfected cells blocks the induction of transcription from the GlcNAc-T V promoter by *neu*. When the rafC4B expression plasmid is replaced by a plasmid expressing a raf double mutant, rafC4Bpm17, which is defective in ras binding in addition to having a defective kinase, the induction by *neu* is unaffected. This result indicates that transduction of the *neu* signal to the GlcNAc-T V promoter is mediated by raf.

Her-*2*/neu *induces increased β(1,6) branching on a set of glycoproteins*

Glycosylation of acceptor oligosaccharides on various glycoproteins by GlcNAc-T V occurs only on a subset of potential acceptors (Do *et al*., 1994). The subset of glycoproteins that are β(1,6)-branched varies among tissues and cell types. Since increases in β(1,6) branching are often observed in oncogenically transformed cells and in human tumors, it is important to determine if glycoproteins that receive β(1,6) branching in non-transformed cells simply show increased levels of branching after transformation, or whether there are unique glycoproteins that are branched only in the transformed cell. In order to identify glycoproteins that carry β(1,6) branches in the cells used in this study, lectin blot analysis using L-PHA was performed on both *neu*-NIH3T3 and NIH3T3 cells after SDS–PAGE. These blots show that there is an increase in the amount of β(1,6) branching after *neu* transfection in most of the glycoproteins that are bound by L-PHA, as would be expected from the increased GlcNAc-T V activity. Most prominent are the lysosomal-associated membrane glycoproteins (LAMPs) which exhibit molecular weights in the range of 90–120 kDa (Saitoh *et al*., 1992) and which can mediate adhesion of cells to the galectins and E-selectin (Do *et al*., 1990; Sawada *et al*., 1994).

Intriguingly, the data also reveal that β(1,6) branches are added selectively to several glycoproteins which appear to show a much greater increase in L-PHA binding after transformation than the increase

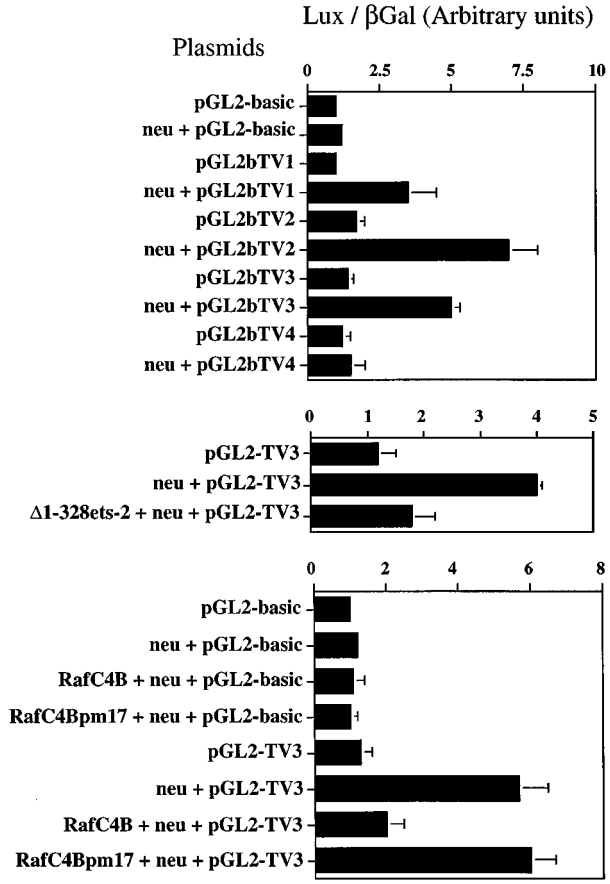

Figure 5 Response of the GlcNAc-T V promoter region to expression of various cDNAs. Upper panel, fragments of the GlcNAc-T V gene respond to *her*-2/*neu*. The *Neu* expression vector was co-transfected into the HepG2 cells with each constructed pGL2-luciferase reporters, pGL2-TV1 through -TV4, as described in Materials and methods. β-Galactosidase expression vectors were included in each transfection, and their expression in each transfection was utilized to normalize the data to control for transfection efficiency. pGL2-Basic is the empty vector with no GlcNAc-T V promoter region. The luciferase activity obtained with the pGL2-Basic was set arbitrarily at one unit. The error bars were calculated from triplicate assays derived from four separate experiments. Middle panel, the GlcNAc-T V promoter activity of fragment TV3 is stimulated by Ets-2. pGL2b-TV3, which contains the shortest GlcNAc-T V promoter sequence tested that responds to *neu* expression, was co-transfected into HepG2 cells with the *neu* expression vector with and without a dominant-negative *Ets*-2 expression plasmid, ets-2 (Δ 1–328). *Neu*, (1/2)*neu* and (1/4)*neu* represent 8 μg, 4 μg and 2 μg of plasmid DNA, respectively. The results were averaged from triplicate experiments. Lower panel, the TV3 promoter activity which responds to *her*-2/*neu* is dependent on *raf*. A similar co-transfection experiment used an expression plasmid containing *raf* and mutants of *raf* instead of Ets-2 and ets-2 (Δ 1–328). RafC4B is a dominant negative form of raf-1. RAfC4Bpm17 contains an inactive point mutation of RafC4B. These experiments were performed twice, with each transfection performed in triplicate. The results from both experiments were averaged and plotted

observed for the LAMPs. For example, a protein with an apparent molecular weight of 156 kDa, denoted by the arrow in Figure 6, shows very low, if any, binding by L-PHA in the NIH3T3 lysate lane. By contrast, in the corresponding area of the blot from the *neu*-NIH3T3 cell lysate, gp156 shows extremely high levels of binding by L-PHA. Silver staining of similar gels reveals that the protein band corresponding to gp156 is a very minor component in both NIH3T3 and *neu*-NIH3T3 cells (data not shown); therefore, its identity can not be easily assigned as yet. Nevertheless, these results show that there are glycoproteins in the oncogenically transformed cell that show high levels of glycosylation by GlcNAc-T V, yet in non-transformed cells are either expressed at very low levels or show insignificant β(1,6) branching.

Discussion

The experiments described in this manuscript show that *neu*, an onocgene of the receptor tyrosine kinase type, affects the expression of cell surface oligosaccharides in a manner similar to those of an earlier study with another tyrosine kinase oncogene, *src*. Both of these oncogenes induce increased synthesis of β(1,6) branched N-linked oligosaccharides by inducing transcription of the GlcNAc-T V gene. The signal from these tyrosine kinases is transduced through the Ras-Raf-Ets pathway. These results are consistent with other studies demonstrating that transfection of a murine mammary carcinoma cell line with H-*ras* caused increased GlcNAc-T V activity and β(1,6) branching (Lu and Chaney, 1993). Also, the Ets family of transcription activators has been shown to enhance GlcNAc-T V expression in a human bile duct carcinoma line (Kang *et al*., 1996).

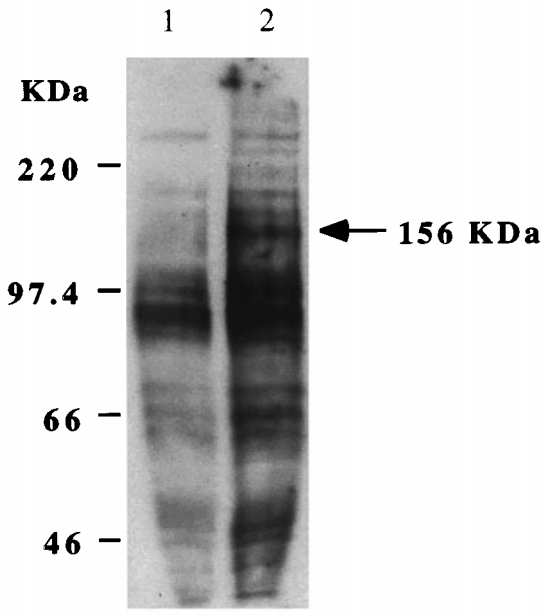

Figure 6 L-PHA staining of a blot of 3T3 and *neu*3T3 cell lysates after SDS–PAGE. NIH3T3 and *neu*-3T3 cells were lysed, supernatants were collected, and identical amounts of protein (20 μg/lane) were loaded on a minigel and subjected to SDS–PAGE. After electro-transfer to Immobilon, the membrane was blocked and probed with L-phytohemagglutinin, followed by an anti-L-phytohemagglutinin horseradish peroxidase conjugate as described in Materials and methods. The ECL reagents were used to detect the peroxidase activity on X-ray film

Many studies have documented the over-expression and amplification of the *her*-2/*neu* oncogene in human breast cancer. This amplification has been postively correlated with the number of lymph node metastases (Borg *et al*., 1991; Pierce *et al*., 1991; Slamon *et al*., 1989). Although the prognostic value of *her*-2 expression has not been fully determined, most studies suggest that over-expression of this oncogene correlates with a poor prognosis (Allred *et al*., 1992; De Potter *et al*., 1990; Tikannen *et al*., 1992). Over-expression of *neu* in the mammary epithelium of transgenic mice resulted in the appearance of tumors that metastasized with high frequency (Guy *et al*., 1992, 1994). These observations are consistent with the fact that *neu* expression is sufficient to induce metastatic potential when expressed in NIH3T3 cells (Yu *et al*., 1992; Yu and Hung, 1991) and in the human breast carcinoma line, MDA-MB-435 (Tan *et al*., 1997). The mechanism by which the *neu* gene induces higher metastatic potential appears to involve the induction of metastasis-associated proteins which modulate the changes of adhesion and invasiveness that are characteristic of the metastatic cascade (Yu *et al*., 1992, 1994). The data presented here suggest that GlcNAc-T V is a member of this class of proteins.

Moreover, several studies have shown that increased β(1,6) branching can cause changes in cellular adhesive properties. Transfection of a cDNA encoding GlcNAc-T V into Mu1Lu cells affected their morphology and adhesive properties to extracellular matrix molecules such as laminin (Demetriou *et al*., 1995). This and other studies have shown that integrins are branched by GlcNAc-T V. Recent results in our laboratories show that when 3T3 fibroblasts are transfected with a GlcNAc-T V cDNA under the control of an inducible promoter and this promoter is activated, the cells show a marked change in morphology with altered homotypic and matrix adhesive properties (Chen, Fregien and Pierce, manuscript in preparation). In addition, the homotypic cell-surface adhesion molecule carcinoembryonic antigen (CEA) is expressed in some colonic adenocarcinomas, and in at least one highly metastatic subline, CEA shows high levels of L-PHA binding (Li *et al*., 1994). Results from experiments that examined the changes in N-linked glycosylation of lysosomal membrane glycoproteins (LAMPs) in sublines of a human colon cancer that showed a range of metastatic potentials correlated higher levels of polyN-acetyllactosamine with increased metastatic potential. This increase was also associated with increased sialylation and lowered fucosylation of the N-linked chains of these glycoproteins. Taken together, these results suggest an association, in both *in vitro* cell transformation systems and in some human malignancies, between more highly branched N-linked chains of particular glycoproteins and a phenotype characterized by altered adhesive properties. The critical hypothesis to now test is whether β(1,6) branching of specific adhesion molecules, such as the integrins or CEA, can affect the binding affinities for their appropriate ligands.

Glycoproteins in transformed cells that express β(1,6) branching may also be useful as markers for transformation, and these glycoproteins are found both

on the cell surface and intracellularly. In the cases of human mammary and colonic epithelial cells, neither of these cell types expresses significant levels of L-PHA binding sites (Li and Roth, 1997). When both of these cell types become carcinomas, they begin to express large amounts of L-PHA binding sites, most likely because of upregulated GlcNAc-T V activity (Fernandes *et al*., 1991; Dennis and Laferte, 1989). A large percentage of these glycoproteins expressing L-PHA binding sites are intracellular, as are those depicted in Figure 1. LAMPs, with molecular weights from 90–120 kDa, make up some of this family of glycoproteins that express β(1,6) branches (Figure 6) and as reported in the study by Saithoh *et al*. (1992), but there are clearly many other glycoproteins in this family.

If any highly branched epithelial glycoproteins are shed from tumor cells into serum, as many glycoproteins in rapidly dividing cells are, it may then be possible to exploit these changes in glycoprotein β(1,6) branching to develop a diagnostic or prognostic serum marker assay for the presence of these carcinomas (Hayes, 1996). Using the *neu*-transformed 3T3 cells as an example, if a very small percentage of transformed 3T3 cells were mixed with a high percentage of non-transformed 3T3 cells and the mixture was grown together in culture, a sensitive assay for the presence of the gp156 (Figure 6) shed into cell culture fluid should allow detection of the presence of the oncogenically transformed cells in the mixture. Studies to test this hypothesis and to identify unique β(1,6) branched glycoproteins in human carcinomas may lead to the development of useful screening reagents for the diagnosis of specific carcinomas.

Materials and methods

Cell lines and plasmids

The *neu*-NIH3T3 cells (B104-1-1 cells) were a kind gift from Dr M-C Hung (Yu and Hung, 1991). Fluorescein-labeled L-PHA and L-PHA were from Vector Labs. Anti-L-PHA horseradish peroxidase conjugates were from EY Labs. GlcNAc-T I and GlcNAc-T V synthetic acceptors were kind gifts from Dr Ole Hinsgaul (Palcic *et al*., 1988). Luciferase substrate and lysis buffer were from Promega. pMMTVneuNT was obtained from Dr William Muller (Muller *et al*., 1988). A dominant-negative form of Raf-1 kinase, RafC4B and a mutated RafC4B, RafC4Bpm17 were kind gifts from Dr Ulf Rapp (Bruder *et al*., 1992). Plasmids pRK5-Ets2 and pRK7 D1-328Ets2 encoding human Ets-2 and its dominant-negative mutant D1-328Ets-2, respectively, were kind gifts from Dr Kim E Boulukos (Aperlo *et al*., 1996). GlcNAc-T V promoter/luciferase reporter plasmids were previously described (Buckhaults *et al*., 1997).

Fluorescence staining

The NIH3T3 and *neu*-NIH3T3 cells were seeded into chamber slides at low density and cultured overnight prior to L-PHA binding. The cells were then fixed with 2% formalin in Hanks' Basic Salts Solution at 37°C for 15 min, permeabilized and blocked with 3% BSA (bovine serum albumin) plus 0.2% saponin in PBS (phosphate-buffered saline) for 15 min at room temperature and then stained with 50 μg/ml fluorescein coupled-L-PHA in the blocking buffer for 1 h. After washing with PBS, the slides were mounted and viewed in a scanning laser confocal microscope.

Enzyme assays

Cells were trypsinized, pelleted and lysed with 50 mM MES pH 6.5, 150 mM NaCl and 1% Triton X-100. Insoluble debris was pelleted in a micro-centrifuge and the supernatants used for the enzyme assays (Shoreibah *et al*., 1992). The assay tubes for GlcNAc-T I contained 1 mM UDP-GlcNAc, 0.5 mM synthetic acceptor, 3×10^5 c.p.m. [^{3}H]-UDP-GlcNAc, 50 mM MES pH 6.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 10 mM NaCl and 0.1% Triton X-100. Five ml of cell lysates was added to each tube and the final volume of each was 10 μl. Assays were done at 37°C for 3 h and stopped with 500 μl cold H_2O. Sep-Pak columns were used to separate the substrates and products as described (Shoreibah *et al*., 1992). The column methanol eluants were counted in a scintillation counter. For the GlcNAc-T V assay, the assay tube contained 0.4 mM synthetic acceptor, 2 mM ADP, 4 mM UDP-GlcNAc and 1×10^6 c.p.m. UDP-[^{3}H]-GlcNAc. Assays were incubated at 37°C for 6 h and products quantitated as described above. Assays were done in duplicate and with at least three protein concentrations. Each cell line was assayed in three separate experiments, and the results were averaged.

RNA gel blot analysis

Total RNA from cells was isolated using the Trizol reagents from BRL-Gibco. Thirty μg total RNA from each cell line was fractionated on a 0.7% formaldehyde gel and blotted to nylon. The blot was hybridized according to the method of Church and Gilbert (1984) with a 1 kb fragment of the rat GlcNAc-T V cDNA that was random-prime labeled with ^{32}P-(Megaprime™ DNA labeling system, Amersham). The hybridization was detected and quantitated using a Phosphorimager.

Transfections

Transfections were performed using the CalPhos Maximizer transfection kit (Clontech) as previously described (Buckhaults *et al*., 1997). Briefly, the day before transfection HepG2 cells were plated into 6-well culture plates at a density of 4×10^5 cells/well. Luciferase reporter plasmid DNAs were used at 1 μg/kb/well; neu expression plasmid DNA at 8 μg/well (except in Figure 5, middle panel); *Ets*-2 and *Raf*-1 expression plasmid constructs were used at 4 μg/well and the internal transfection control, pSV-β-galactosidase plasmid at 2 μg/well. After a 48 h transfection, cells were lysed and assayed for luciferase and β-galactosidase activities. Luciferase activity was then normalized for β-galactosidase activity. All transfections were done in triplicate and the results were averaged.

SDS–PAGE and Western blotting

Cells were lysed, supernatants were collected and 20 μg of protein from each sample were separated by SDS–PAGE and transferred to nylon membranes. After transfer, the membrane was blocked with 3% BSA in PBS and then probed with 10 μg/ml L-PHA followed by 1 : 5000 dilution of anti-L-PHA horseradish peroxidase conjugate. The peroxidase activity was detected using ECL chemiluminescence (Amersham).

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