A STAT-responsive Element in the Promoter of the Episialin/MUC1 Gene Is Involved in Its Overexpression in Carcinoma Cells*

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Ingrid C. Gaemers§§, Hans L. Vos‡‡, Haukeline H. Volders, Sylvia W. van der Valk¶¶, and John Hilkens¶

From the Division of Tumor Biology, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

The mucin-like glycoprotein episialin (MUC1) is highly overproduced by a number of human carcinomas. We have shown previously in a variety of mammalian cell lines that overexpression of this very large transmembrane molecule diminishes cellular adhesion, suggesting that episialin/MUC1 overexpression may play an important role in tumor invasion and metastasis. By using in situ hybridization, we show here that episialin/MUC1 mRNA expression can be increased more than 10-fold in breast carcinoma cells relative to the expression in adjacent normal breast epithelium. In search of the molecular mechanism of this overexpression, we observed that the episialin/MUC1 promoter contains a candidate binding site for transcription factors of the STAT family about 300 base pairs upstream of the transcription start site. Cytokines and/or growth factors such as interleukin-6 or interferon-γ can activate STATs. In the human breast carcinoma cell line T47D, both compounds are able to stimulate transcription of a luciferase reporter gene under the control of a 750-base pair MUC1 promoter fragment proximal to the transcription start site. The observed increase is entirely mediated by the single STAT-binding site, since mutation of this site abolishes stimulation of the reporter by interleukin-6 and interferon-γ. In addition, mutation of the STAT site also decreased the promoter activity in nonstimulated T47D cells, suggesting that the STAT-binding site is among the elements that are involved in the overexpression of MUC1 in tumor cells.

Episialin/MUC11 also known as MUC1, PEM, CA 15-3 antigen, or EMA) is a transmembrane molecule with a large extracellular mucin-like domain. In normal cells episialin/MUC1 is exclusively present at the apical side of the cell, but in carcinoma cells normal polarization is lost and episialin/MUC1 co-localizes with adhesion molecules such as integrins and cadherins. The long and relatively rigid extracellular domain of episialin/MUC1 can shield these adhesion molecules and diminish cellular adhesion, if present at a sufficiently high density on the cell surface (1–3). Overexpression of episialin/MUC1 in carcinoma cells has been frequently reported (4–6) and is expected to have a similar effect on cellular behavior as loss of E-cadherin, the major epithelial cell-cell adhesion molecule, which has been shown to promote invasion and metastasis of carcinoma cells (for review see Refs. 7–9). Therefore, we have proposed that episialin/MUC1 also plays an important role in invasion and metastasis in vivo (10). Indeed, transgenic mice overexpressing episialin/MUC1 develop more aggressive lung tumors than nontransgenic mice,2 whereas episialin/MUC1 null mice show a slower rate of tumor progression (11).

Recent reports have shown that episialin overexpression in various types of neoplasia correlates with poor survival (12–14). Episialin/MUC1 is also the antigen that is measured in the CA 15-3 assay (the main blood marker to detect recurrence of breast cancer), and it is a molecule that is widely considered as one of the most promising molecules to be used for vaccination against breast cancer. Therefore, knowledge about the regulation of episialin/MUC1 overexpression in tumor cells is of utmost clinical importance. In B cell lymphomas it has been shown that episialin/MUC1 overexpression is frequently the result of a t(1;14) (q21;q32) translocation, which brings the episialin/MUC1 gene under the control of an immunoglobulin γ heavy chain enhancer (15, 16) and is consistent with a role in tumorigenesis of these lymphomas. However, the cause(s) of episialin/MUC1 overexpression in carcinoma cells have not been established yet. Previous studies (6, 17) have shown that the episialin/MUC1 mRNA level is severalfold increased in primary breast carcinoma specimens relative to adjacent normal epithelium. However, these results, which were obtained using mRNA blots of tissue homogenates, are inherently imprecise, since both breast carcinoma and normal breast tissue specimens usually contain significant amounts of other nonepithelial cell types. Even so, these results indicate that overexpression of episialin/MUC1 coincides with increased mRNA levels. Episialin/MUC1 mRNA levels in breast carcinoma cell lines also are increased in comparison to immortalized nontransformed epithelial cells (18). The study of Bieche and Lidereau (17) showed that the higher episialin/MUC1 mRNA levels to some degree may be caused by amplification of the MUC1 gene, but additional mechanisms of overexpression clearly are operative as well, since the level of overexpression often is much higher than the level of amplification.

We hypothesized that overexpression of episialin/MUC1 in carcinoma cells is the result of constitutive activation of a transcription factor or inactivation of a repressor.

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1 The abbreviations used are: MUC1, mucin-1; EMSA, electrophoretic mobility shift assay; PBS, fetal bovine serum; ICAM-1, intercellular adhesion molecule-1; IFN, interferon; IL-6, interleukin-6; STAT, signal transducer and activator of transcription; DME, Dulbecco's modified Eagle's medium; bp, base pair; PBS, phosphate-buffered saline.

2 J. Wesseling and J. Hilkens, unpublished data.
Transcriptional Regulation of Episialin/MUC1

Episialin/MUC1 expression is positively regulated by prolactin, which is up-regulated in human breast carcinoma cells. Prolactin binds to the human episialin/MUC1 receptor, and this interaction activates the Stat3 pathway.

Materials and Methods

Cell Lines—T47D cells (human breast carcinoma cell line) were a kind gift from Dr. J. Taylor-Papadimitriou, Imperial Cancer Research Fund, London, UK. A549 (human lung carcinoma cells), HBL-100 (human breast carcinoma cell lines) were obtained from Centocor Inc., Malvern, PA. HLA-60 cells (human promyelocytic leukemia) were obtained from Dr. C. Figdor, Netherlands Cancer Institute, Amsterdam, Netherlands. All cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with penicillin, streptomycin, and 10% fetal bovine serum (FBS).

Antibodies—Monoclonal antibody C-111, directed against amino acids 613–759 of human STAT1, and rabbit polyclonal serum C-20, raised against amino acids 750–769 of mouse STAT3, both from Santa Cruz Biotechnology, were used. The latter antiserum cross-reacts with human STAT3. A rabbit polyclonal antibody specific for the phosphorylated tyrosine (Tyr-705) of STAT3 was obtained from New England Biolabs. Monoclonal anti-STAT2, -3, -5, and -6 from Transduction Laboratories were used to supershift STAT2, -5, and -6. Monoclonal antibodies 139H2 and 214D4 are directed against a peptide epitope in the non-mucin domain of episialin (36).

In Situ Hybridization—In situ hybridization experiments on breast carcinoma cell cultures were performed according to Wilkinson and Nieto (37). The procedures used in this study were also described in a recent publication (34) showing that STAT3 can act as an oncogene.

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The DNA mixture consisted of 0.5 µg of W3b or M3b and 0.01 µg of SV40 Renilla luciferase construct (Promega, control for transfection efficiency) and salmon sperm DNA to a total of 2.5 µg of DNA. The cells were incubated with the DNA/DEAE-dextran mixture until the cells started to round off. Exact incubation conditions varied for each cell line used. T47D and A549 cells were preincubated for 2–4 h with 100 µm chloroquine and were subsequently incubated for 8–12 min with 375 µl of DNA/DEAE-dextran mixture and were subsequently cultured in DMEM without FBS. For the ZR-75-1 cells, the chloroquine treatment was omitted, and incubation with the 375-µl DNA/DEAE-dextran mixture was for 15–20 min, which was followed by a 90-s glycerol shock (15% v/v) in PBS. Subsequently, cells were cultured in DMEM supplemented with 0.25% FBS. The HPAF cells were incubated with 500 µl of DNA/DEAE-dextran mixture for 10–15 min, and a 45-s glycerol shock was applied. Cells were subsequently grown in DMEM with no FBS or 0.25% FBS. Recombinant IFN-γ (Roche Molecular Biochemicals; 200 units/ml) or recombinant IL-6 (Roche Molecular Biochemicals; 400 units/ml) were added 24 h after transfection, and the cells were incubated for an additional 54 h.

Luciferase Assay—Cells were washed with PBS and subsequently lysed at room temperature with 200 µl of Passive Lysis Buffer (Promega) for 20 min. The lysates were collected, and the wells were rinsed with 200 µl of Passive Lysis Buffer. Lysates and wash were combined, and cellular debris was removed by spinning at 14000 rpm in a microcentrifuge for 2 min. The supernatant was stored at −80 °C.

The firefly luciferase reporter and Renilla luciferase (internal control) activities of 15 µl of lysate were measured employing the Dual Luciferase Reagent Assay Kit (Promega) using 75 µl of LAR1 and 75 µl of Stop and Glo buffers. Luminescence was measured using a luminometer (Berthold).

Electrophoretic Mobility Shift Assays (EMSAs)—DNA-binding proteins were extracted basically according to the method of Andrews and Faller (40) with minor modifications. Since the activated (phosphorylated) form of the STATs is predominantly nuclear, additional wash and centrifugation steps were included after the swelling and rupture of the cells to separate the nuclei from the cytoplasmic proteins. Protein yield was determined using the Bio-Rad protein kit.

The double-stranded oligonucleotides were synthesized: wt-MUC1-oligonucleotide, 5′-GGCTATCCGGGAAATGTGT-3′; mutant-MUC1-oligonucleotide, 5′-GGCTACTCGAGAAGTGGT-3′; and ICAM1-oligonucleotide, 5′-GAGGTTCGGGAAGCAGC-3′. The core-binding site of the STATs is underlined. 6 pmol of the double-stranded oligonucleotide was labeled in a final volume of 10 µl using T4 polynucleotide kinase. The oligonucleotide was ethanol-precipitated and redissolved in 100 µl of TE (7.5 µg of nuclear extract (in 1–5 µl depending on the protein concentration) was mixed with 4 µl of 5× binding buffer (20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-Cl, pH 7.5), 1 µg of poly(dI-dC)·poly(dI-dC), and 1 µg of salmon sperm DNA in a final volume of 17 µl (bandshift assay) or 16 µl (supershift assay). This mixture was incubated for 10 min on ice and subsequently 10 min at room temperature. 2 µl of labeled oligonucleotide was added, and the incubation was continued for 30 min at room temperature. In the case of a supershift assay, 1 µl of the antibody preparation was added at the end of this incubation, and the mixture was either left at room temperature for 20 min or on ice for 1 h. In the case of competition experiments, unlabeled oligonucleotide was added during the incubation of the nuclear extract in binding buffer on ice, and the volume of the added water was corrected to keep the final volume constant.

Following incubation, 1 µl of 10× loading buffer (250 mM Tris-HCl, pH 7.5, 0.2% bromphenol blue, 0.2% xylene, cyanol, 40% glycerol) was added to the sample. Samples were analyzed by electrophoresis in 5% polyacrylamide gels for 3 h at 200 V at 4 °C. Running buffer concentrations varied from 0.1× TBE to 1× TBE (1× TBE is 50 mM Tris, 50 mM boric acid, 1 mM Na₂EDTA). 2 µl of the same samples were run separately on similar gels for a shorter period to detect unbound oligonucleotide. After electrophoresis the gels were dried and exposed to Kodak X-OMAT R films at −80 °C.

RESULTS

Overexpression of Episialin/MUC1 in Breast Carcinomas Is Caused by Increased mRNA Expression—The first objective of this study was to obtain unequivocal evidence for an increased expression of episialin/MUC1 mRNA in carcinomas at the single cell level. To this end, we performed immunohistochemical staining and in situ hybridization of serial sections of breast carcinoma specimens using the monoclonal antibody 139H2 directed against the repeat domain of episialin/MUC1 and episialin/MUC1 antisense and sense RNA probes. Subsequently, we compared the expression of episialin/MUC1 RNA and protein in normal and carcinoma tissues in those areas where both cell types were sufficiently close together to be directly comparable in the same microscopic field. Fig. 1 shows a representative picture of the increased protein and RNA levels in two adjacent sections of a mammary carcinoma. The immunohistochemical staining shows only a thin line of apical staining in the normal duct, whereas it is obvious that the apical localization of episialin/MUC1 is lost in the carcinoma cells (Fig. 1A). Another monoclonal antibody, 232A1, which reacts with the nonrepeat domain of episialin/MUC1 and is insensitive to glycosylation, gave very similar results (results not shown). In Fig. 1B the difference between the mRNA levels in normal and carcinoma cells was estimated to be at least 10-fold, by counting the grains over the individual cells. Similar numbers were found in other breast carcinomas. Our in situ hybridization experiments constitute the most direct proof that the overexpression of episialin/MUC1 can indeed be mediated by increased mRNA levels.

A possible explanation for increased episialin/MUC1 mRNA levels may be an increased stability of episialin/MUC1 mRNA in carcinoma cells. Since the mRNA stability cannot be monitored in in vivo or ex vivo samples, we decided to compare previously published data on the stability of episialin/MUC1...
The findings reported above prompted us to study the transcriptional regulation of the episialin/MUC1 gene by some of the known inducers of episialin/MUC1 mRNA and protein, whereas episialin/MUC1 mRNA and protein levels in MCF-7 are high. As shown in Fig. 2, episialin/MUC1 mRNA is very stable in HBL-100 cells. After quantification using a PhosphorImager, we estimate the mRNA half-life by extrapolation of the data to be ~24 h. This is entirely comparable with the half-life in MCF-7 carcinoma cells. Thus a major role for mRNA stability to explain the increased levels of episialin/MUC1 mRNA in carcinoma cells seems unlikely.

The Promoter of Episialin/MUC1 Contains an Active STAT Site—The findings reported above prompted us to study the transcriptional regulation of the episialin/MUC1 gene by some of the known inducers of episialin/MUC1 expression, in particular those that activate STATs. A candidate STAT-binding site (5'-TTCCGGGAAA-3'), which conforms to the consensus TTC-NNGAA (42), has been identified at positions -503 to -495 in the human episialin/MUC1 promoter (30) (numbering also according to this reference). This putative STAT site might well mediate the effects of IFN-γ and/or prolactin, both proven inducers of MUC1 expression, and IL-6 which is often produced by solid tumors, including breast carcinomas (see Introduction). To obtain evidence that this element indeed represents a STAT-binding site, a SacI-Xmn1 promoter fragment spanning nucleotide -722 to +33 and ending in exon 1, 36 nt upstream from the start codon, was cloned in front of the firefly luciferase gene of pGL3-basic resulting in reporter plasmid W3B. In addition, a control construct, M3B, in which the putative STAT site was mutated into 5'-TTACGTAAA-3', was also created (Fig. 3A).

The human mammary carcinoma cell line T47D, a cell line with a high expression level of episialin/MUC1, and the lung carcinoma cell line A549, which shows a low episialin/MUC1 expression, were transiently transfected with both constructs. Both cell lines can be transfected with an acceptable efficiency (~1% of the T47D cells were transfected, which is quite normal for this kind of transfection method). Incubation of transfected T47D cells with IL-6 for 24 h stimulated the wild-type promoter 6–10-fold, whereas the activity of the mutant promoter was hardly affected (Fig. 3B). The activity of the wild-type promoter in A549 cells could also be stimulated by IL-6, although the stimulation was less (Fig. 3C). The response to IL-6 was dose-dependent; optimal stimulation was reached with 800–1600 units/ml (results not shown). For practical reasons all further experiments were carried out with 400 units/ml, which leads to 70% of the maximal response in T47D cells. FBS had little or no effect on the transcriptional activation through this site, because in the presence of 10% FBS the magnitude of induction by IL-6 was the same or slightly lower than in the absence FBS.

In ZR-75-1 breast carcinoma cells and HPAF pancreatic carcinoma cells, both which express high levels of episialin/MUC1, the luciferase activity of the W3B construct was 3–5-fold stimulated, which is similar to the response of the W3B reporter in T47D cells (results not shown). The absolute luciferase activity in these cells, however, was lower than in T47D cells, which may be due to a lower transfection efficiency. When the M3B construct was transfected into ZR-75-1 or HPAF cells, IL-6 had no effect on the luciferase activity.

Interestingly, the wild-type promoter of W3B in T47D cells was consistently about 3-fold more active than the mutant construct in the absence of any growth factor or serum (Fig. 3B), suggesting that one or more STAT factors are constitutively activated in this cell line. As expected, we could not observe a difference between the basal activity of the W3B and M3B constructs in A549 cells, which have a very low episialin/MUC1 expression in contrast to T47D cells (Fig. 3C).

Since IFN-γ has been shown to stimulate episialin/MUC1 expression in ovarian carcinoma cells (19) and is known to stimulate gene expression through STAT1, we investigated whether this cytokine could also activate the episialin/MUC1 promoter via the putative STAT-binding site. To this end, T47D cells, transiently transfected with the W3B and M3B constructs, were treated with IFN-γ for 24 h. Fig. 4 shows that IFN-γ can stimulate the luciferase activity from the W3B construct ~8–10-fold but not from the construct with the mutant STAT site (M3B), suggesting that in addition to IL-6-activated STAT3, STAT1 can also bind to the episialin/MUC1 promoter.

STAT3 and STAT1 Bind to the 5'-TTCCGGGAAA-3' Element in the Episialin/MUC1 Promoter—To prove further that the element targeted in this study is a genuine STAT-binding site and to identify the binding proteins, we performed EMSAs or bandshift experiments, using nuclear extracts from T47D cells. A 19-nt double-stranded oligonucleotide representing the putative STAT site in the episialin/MUC1 promoter was incubated with nuclear extracts from non-induced cells and from cells that had been exposed to IL-6 for 15 min. Pilot experiments had shown that after this incubation time binding to the oligonucleotide was maximal, which is in agreement with data from the literature (43). Nuclear extracts of IL-6-stimulated T47D cells, incubated with the wild-type oligonucleotide, induced the formation of a protein-DNA complex. This complex was neither formed with nuclear extracts from nonstimulated cells nor with an oligonucleotide containing a mutated STAT site (Fig. 5), indicating that the IL-6-induced complex is specific for the putative STAT site.

The protein binding to the putative STAT element after stimulation of T47D cells with IL-6 was positively identified as STAT3 by using a specific antisera in a supershift experiment (Fig. 5). Bandshift and supershift experiments with IL-6-stimulated ZR-75-1 breast carcinoma cells also revealed binding of STAT3 to the STAT element in the episialin/MUC1 promoter (results not shown). The specificity of STAT3 binding to the STAT element in the episialin/MUC1 promoter was shown by competition experiments with excess cold oligonucleotides. The cold episialin/MUC1 wild-type STAT oligonucleotide as well as an oligonucleotide representing the STAT-binding site in the human ICAM1 (CD54) promoter competed the IL-6-induced STAT3 complex formed with the episialin/MUC1 wild-type STAT oligonucleotide (Fig. 5). The "ICAM"
STAT-binding site has a core binding site identical to the episialin/MUC1 STAT-binding site (but different flanking nucleotides) and has been proven to be capable of binding both STAT1 and STAT3 (44, 45). An excess of the mutated episialin/MUC1 STAT oligonucleotide did not compete the IL-6-induced STAT3 complex (Fig. 5C). Moreover, the IL-6-induced protein complex indeed bound to the 32P-labeled ICAM STAT oligonucleotide (results not shown). These results confirm our notion that the episialin/MUC1 promoter contains a genuine STAT-binding site and its flanking sequences (3rd and 4th lanes) and nuclear extracts from untreated T47D cells (1st and 3rd lanes) or from cells treated with IL-6 (400 units/ml) for 15 min (2nd and 4th to 10th lanes). The oligonucleotide-protein complexes were separated on a 5% polyacrylamide gel in 1× TBE. The IL-6-induced STAT-RE binding complex is indicated, whereas an additional, non-identified but specific STAT-RE binding protein complex is designated as non-identified. *, non-specific band; the intensity of this band varied between and even within experiments. The IL-6-induced STAT-RE binding complex could be supershifted with a polyclonal antiserum against STAT3 (5th lane). An excess of either cold wild-type oligonucleotide (10th lane) or cold ICAM-1 oligonucleotide (representing the proven STAT1/STAT3-binding site in the human ICAM-1 promoter, which has the identical core sequence as the episialin/MUC1 STAT-binding site; 5'-GAGGTTCGACAGCAG-3', 8th and 9th lanes) could compete for binding of both the IL-6-induced STAT-RE binding complex as well as the non-identified but specific STAT-RE binding protein complex. An excess of cold mutant oligonucleotide did not compete for binding of these protein complexes (6th and 7th lane). 2 μl of the samples were run separately on similar gels for a shorter time to detect unbound oligonucleotide; this is shown at the bottom of the figure. STAT-RE, STAT response element, 5'-TTCCGGAAA-3'.

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**Fig. 3.** Stimulation of the episialin/MUC1 promoter in T47D breast carcinoma cells and A549 lung carcinoma cells by IL-6. A, the W3B (wild-type promoter in pGL3-Basic) and the M3B (mutant promoter in pGL3-Basic) firefly luciferase reporter constructs and the exact nature of the nucleotide changes in M3B are shown. B and C, the relative luciferase activities of the W3B and M3B constructs in T47D cells (B) and A549 cells (C) in the presence and absence of IL-6 are presented. The assay was carried out with cells that were transiently transfected with 0.5 μg of the W3B or M3B constructs (firefly luciferase), 0.01 μg of the SV40 Renilla luciferase construct, and 1 μg of salmon sperm DNA. Both luciferase activities were measured, and the firefly luciferase activities were normalized using the Renilla luciferase results. All assays were performed in triplicate. Bars indicate the S.D. STAT-RE, STAT response element.

**Fig. 4.** Stimulation of the episialin/MUC1 promoter in T47D breast carcinoma cells by IFN-γ. T47D cells were transiently transfected with the W3B or M3B reporter constructs and stimulated with IFN-γ. For details of the assay see legend Fig. 3. The firefly luciferase results were normalized using the Renilla luciferase activities. The assays were performed in triplicate. Bars indicate the S.D.

**Fig. 5.** Nuclear extracts from IL-6-stimulated T47D cells induce a specific bandshift with an oligonucleotide representing the putative STAT-binding site in the promoter of episialin/MUC1. EMSA using a 32P-labeled 19-nt double-stranded oligonucleotide (WT 5'-GGCTATTCCGGGAAGTGTTT-3') containing the centrally located episialin/MUC1 STAT-binding site and its flanking sequences (3rd to 10th lanes) or a mutant oligonucleotide (MT 5'-GGCTATTCCGGGAAGTGTTT-3'), mutant nucleotides are shown in bold, 1st and 2nd lanes, and nuclear extracts from untreated T47D cells (1st and 3rd lanes) or from cells treated with IL-6 (400 units/ml) for 15 min (2nd and 4th to 10th lanes). The oligonucleotide-protein complexes were separated on a 5% polyacrylamide gel in 1× TBE. The IL-6-induced STAT-RE binding complex is indicated, whereas an additional, non-identified but specific STAT-RE binding protein complex is designated as non-identified. *, non-specific band; the intensity of this band varied between and even within experiments. The IL-6-induced STAT-RE binding complex could be supershifted with a polyclonal antiserum against STAT3 (5th lane). An excess of either cold wild-type oligonucleotide (10th lane) or cold ICAM-1 oligonucleotide (representing the proven STAT1/STAT3-binding site in the human ICAM-1 promoter, which has the identical core sequence as the episialin/MUC1 STAT-binding site; 5'-GAGGTTCGACAGCAG-3', 8th and 9th lanes) could compete for binding of both the IL-6-induced STAT-RE binding complex as well as the non-identified but specific STAT-RE binding protein complex. An excess of cold mutant oligonucleotide did not compete for binding of these protein complexes (6th and 7th lane). 2 μl of the samples were run separately on similar gels for a shorter time to detect unbound oligonucleotide; this is shown at the bottom of the figure. STAT-RE, STAT response element, 5'-TTCCGGAAA-3'.
FIG. 6. IFN-γ induces binding of STAT1 to the episialin/MUC1 promoter. EMSA using 32P-labeled WT episialin/MUC1 STAT-binding oligo and nuclear extracts from either untreated or IFN-γ-stimulated T47D cells (200 units/ml, 30 min). The IFN-γ-induced STAT-RE binding complex is indicated, while an additional, nonidentified but specific, STAT-RE binding protein complex is designated as ‘non-identified’. A supershift with a monoclonal antibody against STAT1 could be observed. Addition of 100-fold excess cold WT-oligo was used as a control for specificity. STAT-RE, STAT response element.

reactive with this protein complex in supershift experiments. Also antibodies against c-Rel, which has been reported to bind to a STAT5-binding site (46), did not supershift. We therefore refer to this band as “non-identified”. EMSAs with A549 extracts did not show the non-identified complex (results not shown).

Similar bandshift and supershift experiments were performed with IFN-γ-stimulated T47D cells. Clear bandshift was observed and a monoclonal antibody against STAT1 revealed a supershifted complex of a slightly higher mobility than the supershifted STAT3-containing complex (Fig. 6). EMSAs with HL-60 cell extracts did not show the non-identified band, and the STAT1 bandshift was clearly observed with extracts of the latter cells stimulated with IFN-γ (Fig. 7). No supershifted complex was detected with anti-STAT1 in EMSAs using IL-6-stimulated T47D extracts. Similarly, no supershift complex was detected with anti-STAT3 in EMSAs using IFN-γ-stimulated T47D or HL-60 extracts (only the results with HL-60 cells are shown in Fig. 7). In conclusion, STAT1 and -3 are capable of binding to the TTCCGGGAA element in the episialin/MUC1 promoter. Thus, in the context of the W3B construct, STAT1 and STAT3 binding most likely leads to the observed up-regulation of the transcription of the luciferase reporter gene upon stimulation with IFN-γ and IL-6, respectively.

Induction of Endogenous Episialin/MUC1 by Cytokines in Cell Lines—Next, we investigated whether IFN-γ or IL-6 could increase episialin/MUC1 expression in various cell lines showing no, intermediate, or high levels of episialin/MUC1. IL-6 increased episialin/MUC1 expression 2.5-fold in A549 cells as measured by FACS experiments (Table I). This induction is comparable with the induction of the reporter gene in the W3B construct (Fig. 3C). However, the induction of the reporter by IL-6 in T47D cells, which show a high basal expression level of episialin/MUC1, was not matched by a comparable increase in the episialin/MUC1 protein levels in these cells (Table I). IFN-γ also hardly stimulated episialin/MUC1 expression in these cells. Similarly, IL-6 or IFN-γ could not induce episialin/MUC1 expression in ZR-75-1 cells, which also express a very high basal level of episialin/MUC1. The MCF-7 cells used in our studies show an intermediate expression level of episialin/MUC1. Episialin expression in this cell line was only slightly enhanced by IL-6 but was 2-fold increased by IFN-γ. These results suggest that the stimulation of episialin/MUC1 expression by the cytokines is moderate and dependent on the expression level in the non-induced state.

Next, we tested whether cytokines could induce episialin/MUC1 expression in an episialin/MUC1 negative non-epithelial cell line. HL-60 promyelocytic cells do not synthesize episialin/MUC1, but expression of several genes can be induced by IFN-γ, including the Fc receptor and MHC molecules (47, 48). Although bandshift experiments revealed that in these cells STAT1 is strongly activated and binds to the STAT site in the promoter of episialin/MUC1, STAT3 antisera and 100-fold excess unlabeled WT-oligonucleotide respectively. A supershift using the antibody against STAT1 could be observed, whereas the anti-STAT3 antibody could not supershift the IFN-γ induced STAT-RE-binding protein complex. STAT-RE, STAT response element.

![Table I](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Relative fluorescence index&lt;sup&gt;a&lt;/sup&gt; (± S.D.)</th>
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<tbody>
<tr>
<td></td>
<td>IL-6</td>
</tr>
<tr>
<td>A549</td>
<td>2.48 (±0.30)</td>
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<tr>
<td>T47D</td>
<td>1.22 (±0.08)</td>
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<tr>
<td>MCF-7</td>
<td>1.28 (±0.02)</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>0.97 (±0.05)</td>
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<sup>a</sup> The fluorescence index (FI) was calculated as (FI − FB)/FB, where FB is the baseline fluorescence intensity of the cells stained with the specific monoclonal antibody 214DA, and FI is the fluorescence intensity of a non-reactive control monoclonal antibody. The relative FI was calculated as (FI of the cells grown with cytokine/FI of cells grown without cytokine). If there is no induction, the relative FI = 1.

<sup>b</sup> The results and S.D. were calculated from three independent experiments.

5 I. Gaemers, unpublished results.
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FIG. 8. Effect of IL-6 on MUC1 mRNA and protein levels in T47D cells. A, total episialin/MUC1 protein levels in either untreated or IL-6-treated T47D cells were determined on Western blot by ECL using the monoclonal anti-repeat antibody 214D4. The position of the 200-kDa marker is indicated; episialin/MUC1 has an apparent molecular mass of over 400 kDa (2 allelic forms). B, Northern blot using mRNA of T47D cells treated with or without IL-6 (400 units/ml, 24 h) and actinomycin D (10 μg/ml, times as indicated). The blot was hybridized with 32P-labeled episialin/MUC1, c-Myc and β-actin cDNAs. The two episialin/MUC1 alleles in T47D differ in size, leading to mRNAs of approx. 4 and 7 kb. The sizes of c-Myc and β-actin mRNAs are respectively 2.4 kb and 1.7 kb. The positions of the 28S and 18S ribosomal bands are indicated. The Northern blot was exposed either to Kodak X-OMAT AR films or to a phospho-imager screen. Quantification was done using a Fuji BAS 2000 phospho-imager and Tina 2.09 software. Quantification of the samples that were not treated with actinomycin D (lanes 1 and 3) showed that IL-6 does not influence steady-state episialin/MUC1 mRNA levels; quantification of the other samples showed that episialin/MUC1 mRNA stability also is not changed by IL-6. The experiment shown is representative for multiple experiments.

DISCUSSION

Immunohistological studies of tissue sections with monoclonal antibodies against peptide epitopes in the mucin domain of episialin suggested that this molecule is strongly overexpressed in breast cancer (4, 5). However, the mucin domain of episialin on normal breast epithelial cells carries numerous branched O-linked carbohydrates that will block or hamper binding of the antibodies, whereas episialin molecules derived from carcinomas mainly contain shorter nonbranched glycans, more easily allowing the access of the antibodies to their epitopes (49–52). Thus, the increased reactivity of most monoclonal antibodies against episialin with tumor cells as reported in the literature could well be explained by differences in post-translational modifications in normal and tumor cells and not necessarily by overexpression of the episialin/MUC1 gene. In this report, our immunohistological studies, employing monoclonal antibodies that are hardly, if at all, affected by O-glycosylation, indicate that overexpression of episialin/MUC1 protein in breast carcinoma cells indeed occurs. The reports of Zaretsky et al. (6) and Bièche and Lidereau (17) indicate that increased episialin mRNA levels are responsible for the overexpression of episialin/MUC1 protein in breast carcinoma cells. However, their studies were performed on whole tissue homogenates and therefore are difficult to interpret. Our in situ hybridization results unequivocally show for the first time that the episialin/MUC1 gene is indeed strongly overexpressed in breast carcinomas relative to normal breast epithelium.

One possible explanation for the increased episialin/MUC1 mRNA levels in carcinoma cells is stabilization of the mRNA. However, the half-life in the normal breast epithelial cell line HBL-100 (Fig. 2) and in the tumor cell lines MCF-7 (41) and T47D (Fig. 8) is not significantly different. In fact, episialin/MUC1 mRNA is very stable, which is compatible with a role of episialin as a structural protein. Other ways to overproduce an mRNA are amplification of the gene and, more rarely, up-regulation of the promoter by mutation. The first mechanism is indeed operative in the case of episialin/MUC1, as shown by Bièche and Lidereau (17), but it is neither able to explain all cases of overexpression nor can it explain the extent of mRNA overexpression in carcinomas and cell lines. A promoter mutation is expected to affect only one of the two alleles, whereas episialin molecules derived from carcinomas mainly contain shorter nonbranched glycans, which can be easily distinguished from one another on the basis of differences in the number of repeats, is directly proportional to the number of gene copies as determined on Southern blots. These observations exclude mutation(s) in the promoter as a mechanism of overexpression and leave changes in transcriptional regulation of the MUC1 gene as the most important mechanism of episialin/MUC1 mRNA overexpression.

Our study shows that the episialin/MUC1 promoter can be stimulated by STAT transcription factors via a STAT-binding site in its promoter. The position and sequence of this site is completely conserved in the promoters of mouse (29) andibbon (28), and the 9-nucleotide core sequence is identical to that of the proven binding site for STAT1 and STAT3 in the ICAM1 promoter (44, 45). We identified the proteins that bind to the STAT site in the episialin/MUC1 promoter as STAT1 in the case of induction by IFN-γ and STAT3 in the case of IL-6. This is completely in line with the current model of IFN-γ and IL-6 signaling (42, 53).

Even without the addition of any growth factor or serum the W3B construct, which contains a firefly luciferase reporter gene driven by 720 bp of the episialin/MUC1 promoter (including the STAT-responsive element), already showed a significant activity in reporter assays in the mammary carcinoma cell line T47D, which decreases 3-fold upon mutation of the STAT site. This observation indicates that a constitutively activated (STAT) factor is present in this cell line, which may contribute to overexpression of episialin/MUC1. Indeed constitutively activated STATs have been reported in breast cancers (32, 33). Moreover, our bandshift experiments do show a specific, STAT response element binding complex that is unaffected by treatment of the cells with IL-6 or IFN-γ. This complex also binds to the ICAM-1 oligonucleotide but is absent in bandshift experiments using an oligonucleotide with a mutated STAT site. The identity of this constitutively activated protein (complex) has not been established yet, but it may affect the overexpression of episialin/MUC1 in carcinoma cells. The latter notion is in line with the observation that neither the non-identified protein complex is present in the low episialin expressing A549 cells nor is there a difference between the basal activity of the W3B and M3B constructs transfected into these cells.

Although a strong induction of the promoter activity by IL-6
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Episialin/MUC1 protein levels do not increase to the same extent when the same cells are treated with IL-6. Since we have found no evidence for post-transcriptional regulatory mechanisms affecting the episialin/MUC1 levels, as for instance has been reported for the rat sialomucin complex (54), this discrepancy must be attributed to either the inability of the promoter to be further stimulated, because it is already fully activated in these cells, or to the presence of the promoter in a truncated form in a plasmid instead of its normal genomic surroundings. Indeed, the level of episialin/MUC1 in the two cell lines showing the highest expression, T47D and ZR-75-1, can barely be induced any further, whereas in cell lines with an intermediate or low expression level, episialin/MUC1 can be up-regulated by either IL-6 or IFN-γ, suggesting that the promoter activity in the former cells has reached its limits. Alternatively, an additional regulatory element located outside the promoter fragment used in the W3B construct may counteract the STAT-induced expression in T47D and ZR-75-1 cells. Preliminary results show that increasing the size of the 5′ promoter fragment in the reporter construct to 2.9 kb (i.e. into the 3′-untranslated sequence of the trypsinobrin 3 gene) does not diminish the ratio between the noninduced and the IL-6-induced luciferase levels.6

Another explanation for the unresponsiveness of the episialin protein levels to induction by IL-6 in some of the IL-6 receptor-positive carcinoma cells may be the inability of the STAT3 promoter to bind to its binding site in the episialin/MUC1 promoter, because the site is already occupied, for instance by the previously mentioned unidentified protein in the bandshifts. Preliminary experiments suggest that this band has a similar or even higher affinity for the oligonucleotide than STAT3. The fact that a STAT3-specific band can be observed in the EMSAs should then be attributed to the presence of excess target oligonucleotide. Induction of the promoter activity in the reporter construct by IL-6 can be similarly explained by the abundance of reporter plasmid in the transiently transfected cells.

Finally, it can be envisaged that by transfecting multiple copies of the promoter plasmid, a repressor of the STAT site is titrated out, resulting in an IL-6-induced expression of the MUC1 promoter in transfected cells only. However, a concentration series of W3B, while keeping the total amount of DNA constant, did not show any evidence for such an effect. Induction of the promoter could be observed with a broad range of W3B DNA concentrations (0.05–2.0 μg of added plasmid, results not shown).

Several other regulatory elements have been identified in the episialin/MUC1 promoter. For instance, Kovarik et al. (30, 55) identified an E box, termed E-MUC1, at position −84 to −74 and a Sp1-like binding site at position −101 to −89. The latter site can bind an inhibitory factor, SpA, or in the absence of SpA the stimulatory factor Sp1. Since SpA and E-MUC1 are expected to be expressed in a tissue-specific fashion, they may confer tissue-specific expression of episialin/MUC1. In line with these results, we found that the presence of activated STAT1, capable of binding to the STAT-binding site in the episialin/MUC1 promoter, does not lead to episialin/MUC1 expression in the promyelocytic HL-60 cells.

Episialin is expressed in activated T cells (56) and plasma cells and not at very low levels during earlier stages of B cell development.7 In addition, STATs are known to regulate various stages of lymphocyte development. Therefore, STAT-de-
A STAT-responsive Element in the Promoter of the Episialin/MUC1 Gene Is Involved in Its Overexpression in Carcinoma Cells
Ingrid C. Gaemers, Hans L. Vos, Haukeline H. Volders, Sylvia W. van der Valk and John Hilkens


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