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Induction of MUC2 and MUC5AC Mucins by Factors of the Epidermal Growth Factor (EGF) Family Is Mediated by EGF Receptor/Ras/Raf/Extracellular Signal-regulated Kinase Cascade and Sp1*

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The 11p15 mucin genes (MUC2, MUC5AC, MUC5B and MUC6) possess a cell-specific pattern of expression in normal lung that is altered during carcinogenesis. Growth factors of the epidermal growth factor family are known to target key genes that in turn may affect the homeostasis of lung mucosae. Our aim was to study the regulation of the 11p15 mucin genes both at the promoter and protein levels to assess whether their altered expression may represent a key event during lung carcinogenesis. Studies were performed in the mucopidermoid NCI-H292 lung cancer cell line. Cell treatment with epidermal growth factor (EGF), transforming growth factor α (TGF-α), or tumor necrosis factor α (TNF-α) resulted in a dramatic increase of MUC2 and MUC5AC mRNAs levels, promoter activity, and apomucin expression, whereas those of MUC5B and MUC6 were unchanged. pGL3 deletion mutants of MUC2, MUC5AC, and MUC5B promoters were constructed and used in transient transfection assays to characterize EGF- and TGF-α-responsive regulatory regions within the promoters. They were located in the −2627/−2097 and −202/−1 regions of MUC2 and MUC5AC promoters, respectively. Finally, we demonstrate that transcription factor Sp1 not only binds and activates MUC2 and MUC5AC promoters but also participates to their EGF- and TGF-α-mediated up-regulation. We also show that Sp3 is a strong inhibitor of 11p15 mucin gene transcription. In conclusion, MUC2 and MUC5AC are two target genes of EGFR ligands in lung cancer cells, and up-regulation of these two genes goes through concomitant activation of the EGFR/Ras/Raf/Extracellular Signal-regulated Kinase-signaling pathway and Sp1 binding to their promoters.

Mucins have been postulated to be important molecules in maintaining epithelial homeostasis in inflammatory diseases and cancer in that they are large O-glycoproteins expressed either at the cell surface or as secreted molecules to form a protective gel. Mucin genes MUC2, MUC5AC, MUC5B, and MUC6 are clustered on the p15 arm of chromosome 11 (1) and encode large secreted O-glycoproteins that participate in mucous formation and epithelium protection (2, 3). However, their precise biological role as key genes during sequential steps of lung carcinogenesis has yet to be proven.

In the surface epithelium of the respiratory tract, MUC5AC is exclusively expressed in mucus-secreting goblet cells, whereas MUC1 and MUC4 are expressed in all epithelial cells. MUC5B is prominent in the mucous cells of the submucosal glands, and MUC2 is weakly expressed in both cell types (4–6). In epithelial lung diseases this pattern of expression is altered and has been correlated to poor prognosis of the tumor (invasiveness, metastasis) (7). For example, aberrant expression of MUC6 in bronchiopulmonary adenocarcinoma, loss of expression of MUC5AC and MUC5B in epidermoid carcinoma, and a strong expression of the four 11p15 mucin genes in bronchioalveolar carcinoma was recently described (5).

Growth factors are thought to be involved in goblet cell production because hypersecretory diseases are associated with abnormal epithelial cell growth and differentiation, and epithelium wounding leads to repair and remodeling processes (8). More recently, Nadel and co-workers (9, 10) focused on MUC5AC regulation by epidermal growth factor (EGF)1 in the lung. They showed that MUC5AC mRNA expression was increased after ligand binding to the EGFR and activation of the mitogen-activated protein kinase cascade. Positive correlation between EGFR and MUC5AC expression and localization in goblet cells of human bronchi is also in favor of the activation of a series of events involving EGFR followed by activation of MUC5AC expression in lung cells (11). However, these studies only focused on MUC5AC, whereas it has been shown by our laboratory that the expression of the other 11p15 mucin genes is also impaired in lung diseases (5, 6). Moreover, despite these promising studies, no data regarding the molecular mechanisms responsible for mucin gene regulation at the promoter level are available. Their identification remains a big challenge but is mandatory if one wants to demonstrate whether mucins are indeed key molecules in carcinogenesis.

At this time, promoter structures of MUC2 (12, 13),

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1 The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal-regulated kinase; RT, reverse transcriptase; TGF-α, transforming growth factor-α; TNF-α, tumor necrosis factor-α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kb, kilobases.
MUC5AC (14) and MUC5B (15, 16) are known. All of them are flanked by TATA boxes, and upstream cis-elements that confer cell-specific expression to the genes were described. MUC2 promoter is GC-rich over a very long stretch (4 kb) and, as expected, was shown to be regulated by ubiquitous transcription factor Sp1 (12, 17). MUC5B promoter is characterized by two transcription units (15, 16). The proximal unit is also regulated by Sp1/Sp3 factors, whereas the distal unit is under the regulation of ATF/CREB transcription factors. The promoter of MUC5AC is only known over 1.4 kb (14). It is characterized by putative binding sites for Sp1, AP-2, and NF-κB transcription factors and key cis-elements, yet to be described, operate within 4 kb upstream of the transcription start site (14).

Our aim in the laboratory is to better understand transcriptional regulation of 11p15 mucin genes to propose new therapeutic targets in epithelial diseases (inflammation and cancer) (18). In this study, regulation of the four 11p15 mucin genes MUC2, MUC5AC, MUC5B, and MUC6 was studied for the first time simultaneously at the mRNA, promoter, and protein levels. We focused on EGF- and TGF-α-mediated regulation of these genes due to the importance of these growth factors both during lung development (19) and carcinogenesis (8). Moreover, because it was previously shown for other genes, such as gastrin (20) or apolipoprotein A-I (21), that Sp1 may participate to EGF-mediated up-regulation of the target gene, we looked at the Sp1 effect on mucin gene regulation. We demonstrate that among the four 11p15 mucin genes only MUC2 and MUC5AC are regulated at the transcriptional (promoter activity and mRNA levels) and protein levels by EGF and TGF-α and that the activated signaling pathway goes through Ras/Raf/ERK kinases. Finally, we show that Sp1 not only transactivates MUC2 and MUC5AC transcription but also participates in their up-regulation by EGF and TGF-α.

**Immunohistochemistry**—Confluent cultures were trypsinized, centrifuged, washed once with 1x phosphate-buffered saline before fixation of the pellet in 4% formaldehyde, and embedding in paraffin. 3-μm sections were prepared, and morphological analysis was done after hematoxylin-eosin-safran/Astra blue staining. Pretreatment and immunostaining of the sections were performed as described before (22). Polyclonal anti-MUC6, anti-MUC2, anti-MUC5AC, and anti-MUC5B antibodies were a kind gift from Dr. Carlstedt (University of Lund, Lund, Sweden) (23–26). Anti-Ck7 antibody (clone OV-TL 12/30) was purchased from Dako.

**Plasmid Constructions**—The MUC5B-pGL3 deletion mutants (2140, 1599, 1596, 1896) covering 2044 nucleotides upstream of the first ATG were previously described in Van Seuningen et al. (15) and Perrais et al. (16). MUC5AC promoter was obtained by running PCRs on human genomic DNA using the following pair of oligonucleotides: forward primer, 5′-CTA GCC ACC TGT GGG AAG CAA G-3′; and reverse primer, 5′-CTT CCT CCG GCC AAC ACT CAT TGG GTG GAC-3′. PCR conditions with Taq polymerase (2 units, Roche Molecular Biochemicals) and 20 ng of DNA were 4 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 64°C, 2 min at 72°C and then 2 min at 72°C. The PCR products obtained in these conditions covers 1.36 kb of the sequence published by Li et al. (14). MUC2 promoter was obtained by running PCR on the cosmide clone ELO19 isolated from a pWE15 library previously used in the laboratory to isolate the MUC5AC gene (27). PCR on ELO19 was performed with 20 ng of DNA and with forward primer, 5′-GGG AGC TCC TGG TGA TGC AGG TTT GTG AT-3′, and reverse primer, 5′-GGA CGG GTG ACC CCC ATG CAT TGG GTG GAC-3′. PCR was carried out with 2.5 units of Taq polymerase (Qiagen) without buffer as follows. Cycles were followed by 35 cycles of 30 s at 94°C, 30 s at 58°C, and 2 min at 72°C and then 15 min at 72°C. The PCR product obtained covers 2.8 kb of the sequence published by Gum et al. (12). Both PCR products were then subcloned into pCR2.1 vector (Invitrogen) before subcloning into SacI-MluI sites of the promoterless pGL3 Basic vector (Promega). Internal deletion mutants (MUC2 1852, 2214, and 2144 and MUC5AC 2144, 2189, and 2214) were generated by PCR using pairs of primers bearing specific restriction sites at their 5′ and 3′ ends (Table I). PCR products were digested, gel-purified (Qiagel quick extraction kit, Qiagen), and subcloned into the pGL3 Basic vector (Promega) that had been previously used with the same restriction enzymes. All clones were sequenced on both strands on an automatic Li-COR sequencer (ScienceTec, Les Ulis, France) using infrared-labeled RV3 and GL2 primers (Promega). Plasmids used for transfection studies were prepared using the Endofree plasmid Mega kit (Qiagen).

**RT-PCR**—Total RNAs from NCI-H292 cells were prepared using the QIAamp RNA blood mini-kit from Qiagen. Total RNA (1.5 μg) was used to prepare cDNA (Advantage RT-for-PCR kit, CLONTECH). PCR was performed on 5 μl of cDNA using specific pairs of primers as described previously (15). Primers were: MUC2 forward primer (5′-CCGAAGCTTACGACGGCATCATG-3′) and reverse primer (5′-CGCCAGGCTGACAGGAGGATCCCCG-3′) (nucleotide positions 15291–15312 and 15667–15669, respectively accession number L21998) (28); MUC5AC forward primer (5′-GATCACCACGGCATCATG-3′) and reverse primer (5′-CCGAAGCTTACGACGGCATCATG-3′) (nucleotide positions 2897–2917 and 3284–3305, respectively, accession number AJ011402/16), MUC5B forward primer (5′-CTCGGAAGGGCTGAGACAGCCG-3′) and MUC5B reverse primer (5′-GGGCG-
TABLE II
Sequences of the sense oligonucleotides used for gel shift assay experiments

| Oligonucleotide name | Sequence (5’ → 3’) |
|----------------------|-------------------|
| T43 (Spl site), −87/−73 | CACGGCCCCGGCCT |
| T44 (CACCC box), −74/−57 | TGCCCCACCCAGTGGAAG |
| T46 (Spl site), −231/−211 | ATGTGGGAGGACCCCTGCA |

AGCCAGGAGACGAGG-3’ (nucleotide positions 9057–9078 and 10108–10127, respectively, accession number Y09788) (29); MUC6 forward primer (5’-GATGGCAGGTGAGGGGAGTTA-3’ and MUC6 reverse primer (5’-GCGAGGTCCGCTTCTTGGGA-3’) (nucleotide positions 1034–1054 and 1433–1454, respectively, accession number U97698) (30). GAPDH was used as an internal control. MUC2, MUC5AC, MUC5B, MUC6, and GAPDH PCR product sizes are 401, 409, 415, 421, and 980 base pairs, respectively.

Transfections—Transfections and co-transfections were performed using Effectene™ reagent (Qiagen), and luciferase activities were measured as previously described (15, 16). Total cell extracts were prepared after a 48-h incubation at 37 °C using 1× lysis reagent buffer (Promega) as described in the manufacturer’s instruction manual. Total protein content in the extract (4 µl) was measured using the bicinchoninic acid method in 96-well plates as described in the manufacturer’s instruction manual (Pierce). Relative luciferase activity was expressed per µg of protein. Each plasmid was assayed in triplicate in at least three separate experiments.

Nuclear Extract Preparation—Nuclear extracts from NCI-H292 cells were prepared as described by Van Seuningen et al. (31) and kept at −80 °C until use. Protein content (2 µl of cell extracts) was measured using the bicinchoninic acid as described above.

Oligonucleotides and DNA Probes—The sequences of the oligonucleotides used for gel shift assays are indicated in Table II. They were synthesized by MWG-Biotech (Germany). Equimolar amounts of single-stranded oligonucleotides were annealed and radiolabeled using T4 polynucleotide kinase (Promega) and [γ-32P]dATP. Radiolabeled probes were purified by chromatography on a Bio-Gel P-6 column (Bio-Rad, Ivry-sur-Seine, France).

Electrophoretic Mobility Shift Assays—Nuclear proteins (5 µg) were preincubated for 20 min on ice in 20 µl of binding buffer with 2 µg of poly(dI-dC) (Sigma) and 1 µg of sonicated salmon sperm DNA. Radiolabeled DNA probe was added (120,000 cpm), and the reaction was left for another 20 min on ice. For supershift analyses, 1 µl of the antibody of interest (anti-Sp1, anti-Sp3, Santa-Cruz Laboratories, Tebu, France) was added to the proteins and left for 1 h on ice before adding the radiolabeled probe. Reactions were stopped by adding 2 µl of loading buffer. The mixtures were loaded onto a 4% non-denaturing polyacrylamide gel, and electrophoresis conditions were as described in Van Seuningen et al. (15). Gels were vacuum-dried and autoradiographed overnight at −80 °C.

RESULTS
Expression of 11p15 Mucins in NCI-H292 Cells—Expression of 11p15 mucin genes was studied by RT-PCR. As shown in Fig. 1, NCI-H292 cells express MUC2, MUC5AC, and MUC5B mRNAs, whereas MUC6 mRNA is not detected (lane 1). Because NCI-H292 cells are described as a mucoepidermoid cell line, we looked at CK7 and mucin expression by immunohistochemistry using specific antibodies (Fig. 2). CK7, a marker characteristic of a glandular phenotype, is expressed in 100% of the cells (Fig. 2B); MUC2 (Fig. 2C) and MUC5AC (Fig. 2D) apomucins are expressed in 20–25% of the cells, respectively, MUC5B and MUC6 apomucins were not detected in these cells (not shown). In Alcian blue-stained cells, a few grains of secretion could be detected (Fig. 2A). In conclusion, mucoepidermoid NCI-H292 cells express MUC2, MUC5AC, and MUC5B at the mRNA level, but only MUC2 and MUC5AC apomucins are translated and expressed in the cells.

EGF and TGF-α on mucin expression both at the mRNA and protein levels. By RT-PCR (Fig. 1), we show that treatment of the cells with EGF (lane 2) or TGF-α (lane 3) results in a dramatic increase of MUC2 and MUC5AC mRNA levels. Because EGFR is known to be up-regulated by the pro-inflammatory cytokine TNF-α, which is increased in hypersecretory diseases (8), the synergistic effect between TNF-α and EGF or TGF-α was tested. TNF-α up-regulates MUC2 and MUC5AC expression level (lane 4), but no synergistic effect was observed between TNF-α and EGF (lane 5) or TGF-α (lane 6). The different treatments had no effect on MUC5B mRNA levels in these cells, and MUC6, which is not expressed in this cell line, was not induced either.

The up-regulation of MUC2 and MUC5AC mRNA levels was confirmed at the protein level by immunohistochemistry. As shown in Fig. 2E, a dramatic increase in MUC2 apomucin expression was observed as 90–100% of the TGF-α-treated cells express MUC2 compared with 20% in untreated cells (Fig. 2C). MUC5AC expression increased to 30% (Fig. 2F) when only a few cells were stained in untreated cells (Fig. 2D). No staining was observed with anti-MUC5B and anti-MUC6 antibodies (data not shown). The same results were obtained when cells were treated with EGF. We can, thus, conclude that MUC2 and MUC5AC up-regulation by EGF and TGF-α in NCI-H292 cells most likely involve regulatory mechanisms at the transcriptional level.

Identification of EGF- and TGF-α-responsive Regions within MUC2 and MUC5AC Promoters—To do that, we first undertook to identify the regulatory regions within the promoters that are responsive to EGF and TGF-α. For these studies, a panel of deletion mutants covering promoter regions of MUC2, MUC5AC, and MUC5B were constructed in promoterless pGL3 basic vector. Cells were then transfected with the different deletion mutants and treated with the growth factor of interest in the same conditions as in Fig. 1. Regarding MUC2 promoter (Fig. 3A), EGF and TGF-α selectively increased luciferase activity of the longest fragment, which covers the −2627/−1 region of MUC2 promoter (1852; 3- and 2.5-fold activation, respectively). No effect was seen on fragments covering the −2096/−27 (2214) and −947/−1 (1824) regions, indicating that the −2627/−2097 region of MUC2 promoter is necessary to observe a response to EGF or TGF-α. Regarding MUC5AC promoter (Fig. 3B), EGF and TGF-α increased luciferase activity of the three fragments tested (2-3-fold activation). The
region −202/−1 is thus sufficient to get a response, but cooperative elements are present further upstream (−1366−487) since a significant increase is observed when using the longest deletion mutant (compare black bars with gray bars).

Having shown that treatment of NCI-H292 cells with TNF-α resulted in an increase of MUC2 and MUC5AC mRNA levels (Fig. 1, lane 4), we also looked at its effect on their promoters. As shown in Figs. 3 and 4, TNF-α did not have any significant effect of MUC2 and MUC5AC promoter activity. As expected, no synergistic effect between TNF-α and EGF or TGF-α was observed on either promoter, thus confirming RT-PCR results (see Fig. 1, lanes 5 and 6). Finally, the different treatments had no effect on the two promoters of MUC5B (proximal and distal, Fig. 3C), those results being in agreement with RT-PCR data (see Fig. 1). From these studies it can be concluded that EGF and TGF-α activate MUC2 and MUC5AC promoter activity and that responsive elements are present in the −2627/−2097 and −1366/−1 regions of MUC2 and MUC5AC promoters, respectively. In the experiments that follow we, thus, focused our work on the regulation of these two genes.

**EGF, TGF-α, and TNF-α Stimulate MUC2 and MUC5AC Gene Expression at the Transcriptional Level**—To further identify the intracellular mechanisms used by EGFR ligands and TNF-α that lead to a higher expression of MUC2 and MUC5AC in NCI-H292 cells, a 30-min pretreatment with actinomycin D, a transcriptional inhibitor, or cycloheximide, an inhibitor of protein synthesis, before exposure to EGF, TGF-α, or TNF-α, was applied. As shown in Fig. 4, no effect of actinomycin D and cycloheximide is seen on the basal level of MUC2 or MUC5AC mRNAs in these cells (lanes 1–3). On the other hand, actinomycin D treatment completely blocked the EGF-, TGF-α-, and TNF-α-mediated up-regulation of MUC2 and MUC5AC (lanes 6, 9, and 12). In the same conditions, cycloheximide had no effect (lanes 5, 8, and 11). Thus, these data indicate that EGF, TGF-α, and TNF-α increase MUC2 and MUC5AC expression at the transcriptional level and that activation does not require de novo protein synthesis.

**EGF Ligands Stimulate MUC2 and MUC5AC via EGFR/Ras/Raf/ERK-signaling Cascade**—To determine which signaling pathway is induced by EGF or TGF-α, three pharmacological agents were used in inhibition studies. Tyrophostin AG1478 is a selective inhibitor of EGFR tyrosine kinase activity, radicicol selectively depletes cells in Raf kinase (33), PD98059 is a mitogen-activating protein/ERK kinase 1 (MEK-1) inhibitor, and U0126 is an inhibitor of MEK-1 and MEK-2. As a control, cells were treated with these inhibitors with no further treatment. As shown in Fig. 5A, these inhibitors had no effect on the basal expression of MUC2 and MUC5AC mRNAs (compare lanes 2–4 to lane 1). On the contrary, pretreatment with tyrophostin AG1478 led to a complete inhibition of MUC2 and MUC5AC gene up-regulation by EGF or TGF-α (compare lanes 6 and 10 to lanes 5 and 9, respectively). Pretreatment with radicicol (lanes 7 and 11), PD98059 (lanes 8 and 12), or U0126 (data not shown) partially inhibited the response to EGFR ligands. Thus, these results indicate that EGF- and TGF-α-mediated up-regulation of MUC2 and MUC5AC in NCI-H292 cells goes through a signaling pathway involving sequential activation of EGFR kinase, Ras/Raf, and ERK but that most likely another signaling pathway is involved since inhibition by radicicol, PD98059, and U0126 is only partial.

In functional studies, in which transfected cells were pretreated with tyrophostin AG1478 or radicicol in the same conditions, a total inhibition of the EGF- and TGF-α-mediated up-regulation of MUC2 and MUC5AC deletion mutants was observed (Fig. 5B). These results indicate that the responsive elements to the activated EGFR/Ras/Raf/ERK cascade are present in the −2627/−1 and −1366/−1 regions of MUC2 and MUC5AC promoters, respectively.

**Binding of Transcription Factors Sp1 and Sp3 to MUC5AC Promoter**—Transcription factors of the Sp family are important regulators of MUC2 and MUC5B mucin gene expression (12, 15–18) and are known to interfere with EGF-mediated regulation of numerous genes (34). The high density of GC-rich boxes upstream of the transcriptional start site of MUC5AC is also in favor of such a regulatory role for Sp1 on that gene. However no data regarding regulation of MUC5AC by Sp1/Sp3 has been published so far. Electrophoretic mobility shift assays studies were thus carried out to identify new Sp1/Sp3 binding sites within MUC5AC promoter. Two shifted bands were visualized with probe T44 (Fig. 6, lane 2). This probe contains a CACCC box binding site located at −74/−57 in MUC5AC promoter. Upon the addition of anti-Sp1 antibody (lane 3), no effect is seen, whereas the addition of anti-Sp3 antibody resulted in a decrease of the band intensities (lane 4). Thus, complexes 1 and 2 correspond to the two forms of Sp3 (70 and 105 kDa). T43

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**Fig. 2. Study of the expression of MUC2 and MUC5AC apomucins in NCI-H292 cells and effect of TGF-α on their expression by immunohistochemistry.** Untreated cells (A–D) and TGF-α-treated cells (20 ng/ml, 24 h) (E and F) are shown: A, Alcian blue staining; B, CK7 expression; C and E, MUC2 expression; D and F, MUC5AC expression. Immunostaining protocol is described under “Materials and Methods.” Magnification ×200.
covers the nucleotides −87−73 of MUC5AC promoter and contains a Sp1/Sp3 putative binding site. When incubated with nuclear proteins, three shifted bands were visualized (lane 6; complexes 1, 2, and 3). Complex 3 corresponds to Sp1 binding since a supershift occurred upon the addition of the anti-Sp1 antibody in the mixture to produce complex 5 (lane 7). Complexes 1 and 2 correspond to the two forms of Sp3 (lane 6), since these two bands were supershifted upon the addition of the anti-Sp3 antibody to produce complex 4 (lane 8). T46, which is representative of another Sp1/Sp3 binding site at 231−211 in MUC5AC promoter, also binds the two transcription factors (lanes 9−12). Altogether, it can be stressed from these experi-

FIG. 3. Effect of EGF, TGF-α, and TNF-α on MUC2, MUC5AC, and MUC5B. A, pGL3 deletion mutant transcriptional activity in NCI-H292 cells is shown. Schematic representations and localization of the different pGL3-mutants covering 2627 nucleotides of MUC2, 1366 nucleotides of MUC5AC (B), and 2044 nucleotides of MUC5B (C) promoters with Sp1/Sp3 putative binding sites are shown on the left part of the figure. Luciferase diagrams are on the right-hand side. REF. refers to the normalized luciferase activity of the fragment without any treatment.

FIG. 4. Study of the effect of cycloheximide and actinomycin D on EGF-, TGF-α-, and TNF-α-mediated up-regulation of MUC2 and MUC5AC in NCI-H292 cells by RT-PCR. Cells were pretreated with cycloheximide (20 μg/ml) or actinomycin D (1 μg/ml) for 30 min (lanes 1−3) before EGF (lanes 4−6), TGF-α (lanes 7−9), or TNF-α (lanes 10−12) treatment. GAPDH was used as an internal control. PCR products were separated on a 2% agarose gel run in 1× Tris-buffered EDTA buffer in the presence of ethidium bromide.
FIG. 6. Identification of Sp1 and Sp3 cis-elements in MUC5AC promoter by electrophoretic mobility shift assays. 5 μg of nuclear extracts prepared from NCI-H292 cells were incubated with the radiolabeled DNA probes as indicated. Lanes 1–4, T44, Sp1-binding site at 74/−57; lanes 5–8, T43, CACCC box at 87/−73; lanes 9–12, T46, Sp1 binding site at 231/−211. Supershift experiments (ss-) were carried out by adding 1 μl of the antibody of interest (lanes 3, 7, and 11, anti-Sp1; lanes 4, 8, and 12, anti-Sp3). Radiolabeled probe alone was loaded in the first lane of each series (lanes 1, 5, and 9). DNA-protein complexes with Sp1 (complex 3) and Sp3 (complexes 1 and 2) and supershifted complexes with anti-Sp1 (complex 5) and anti-Sp3 (complex 4) are indicated by arrows. An asterisk indicates the position of the supershift obtained with anti-Sp3 antibody (lanes 8 and 12), which is located just above the DNA-protein complex obtained with Sp1.

FIG. 5. Effect of tyrphostin AG1478, radicicol, and PD98059 on MUC2 and MUC5AC expression in NCI-H292 cells. A, effects on mRNA levels were measured by RT-PCR. Cells were pre-treated with tyrphostin AG1478 (10 μM), radicicol (10 μM), or PD98059 (30 μM) for 30 min (lanes 1–4) before EGF (lanes 5–8) or TGF-α (lanes 9–12) treatment. GAPDH was used as an internal control. PCR products were separated on a 2% agarose gel run in 1× Tris-buffered EDTA buffer in the presence of ethidium bromide. B, effects on promoter activity was studied by transient transfection assays in the same conditions. REF: refers to the normalized luciferase activity of the fragment without any treatment.
that Sp1 and Sp3 engage with two and three cognate cis-elements within the first 300 bp of MUC5AC promoter, respectively.

Role of Transcription Factors Sp1 and Sp3 on MUC2 and MUC5AC Promoter Activity—Knowing that Sp1 and Sp3 interact with their cognate cis-elements within MUC2 (17) and MUC5AC (this report) promoters, co-transfection studies were then performed to study their biological effect on MUC2 and MUC5AC transcription activity. pGL3-MUC2 and pGL3-MUC5AC deletion mutants were transfected in the presence of expression vectors encoding either Sp1 (pCMV-Sp1) or Sp3 (pCMV-Sp3) (Fig. 7). Empty pCMV4 vector was used as the reference (gray bar). Co-transfection studies performed on MUC2 indicate that Sp1 strongly transactivates the -947/-1 region of the promoter (black bars, 5-fold), whereas a weak effect is observed on the two other fragments tested (2-fold). Sp3 inhibits MUC2 promoter activity (white bars, 25–50% inhibitory effect) and competes away Sp1 transactivating effect (dark gray bars). MUC5AC is strongly transactivated by Sp1 throughout the 1.4 kb of the promoter region (black bars, 6–8-fold induction), whereas Sp3 has no effect (white bars). As for MUC2, Sp3 competed away Sp1 positive regulatory effect on MUC5AC promoter activity (dark gray bars). From these studies, it can be concluded that ubiquitous transcription factors Sp1 and Sp3 are important regulators of MUC2 and MUC5AC transcription with positive and negative regulatory effects, respectively.

Sp1 Acts as a Mediator in EGF- and TGF-α-mediated Up-regulation of MUC2 and MUC5AC—Having shown that Sp1 binds and transactivates MUC2 and MUC5AC promoters, we then undertook to study whether it mediates EGF/TGF-α activation of these two genes. To this end, we used mithramycin A, a drug that modifies GC-rich regions of the DNA and blocks Sp1 binding. As shown in Fig. 8A, inhibition of Sp1 binding by mithramycin A dramatically reduced MUC2 mRNA levels in the cells, whereas the effect was not as pronounced on MUC5AC (compare lane 2 with lane 1). More interestingly, pretreatment of the cells with mithramycin A before EGF or TGF-α treatment significantly decreased the EGF (compare lane 4 with lane 3)- and TGF-α (compare lane 6 to lane 5)-mediated up-regulation of MUC2 and MUC5AC mRNA levels. Again, the inhibitory effect was more pronounced on MUC2.

In functional studies, similar effects were observed on MUC2 and MUC5AC basal transcriptional activities, thus confirming RT-PCR data. As shown in Fig. 8B, cells treated with mithramycin A had a much lower basal activity of MUC2 promoter (fragment 1852, 50% inhibition). The effect on MUC5AC was much weaker and restricted to the first 202 nucleotides of the promoter (fragment 2113; 25% inhibition). The role of Sp1 as a mediating agent in TGF-α-mediated up-regulation of MUC2 and MUC5AC promoters was also studied by means of transfections. The results shown in Fig. 8C confirm the data obtained by RT-PCR. Indeed, transfected cells pretreated with mithramycin A before TGF-α incubation resulted in a total loss of MUC2 and MUC5AC promoter activity (black bars) when compared with TGF-α treated cells (white bars). The same results were obtained with EGF (not shown). Altogether these results demonstrate that Sp1 binding is essential for MUC2 and to a lesser extent for MUC5AC basal expression and that it also participates to the EGF- and TGF-α-mediated up-regulation of these two genes.

**DISCUSSION**

Organization, evolution, and regulation of the four 11p15 mucin genes, MUC2, MUC5AC, MUC5B, and MUC6 have been extensively studied in our laboratory (1, 4, 5, 15, 16, 18, 35–37). *In situ* hybridization and promoter functional studies conducted by us and others indicate that they are tightly regulated (2, 17) and have an altered profile of expression in human diseases of the respiratory, gastrointestinal, and urogenital tracts (2, 5, 38–42). Such alterations in expression (overexpression, repression, neoexpression) is usually characteristic of genes playing important roles in carcinogenesis.

Mucin hypersecretion and goblet cell hyperplasia are characteristic of inflammatory diseases and carcinogenesis of the lung (37, 40, 43, 44). Besides inflammatory mediators, growth factors could be involved in goblet cell production, because hypersecretory diseases are associated with abnormal epithelial cell growth and differentiation, and epithelium wounding leads to repair and remodeling processes (8). It has been postulated that EGF and its receptor EGFR could be good candidates. In human airways, EGFR is expressed in the fetus, where it is involved in cell proliferation, branching, morphogenesis and epithelial cell differentiation (19). Its expression is sparse in the adult lung but is expressed in lung tumors and asthma and is up-regulated by the pro-inflammatory cytokine TNF-α. Furthermore, K-Ras mutations are frequent in mucinous bronchioalveolar carcinoma and often associated with mucinous differentiation (45).

In this paper, we thus undertook to study the intracellular molecular mechanisms induced by EGF and TGF-α alone or in synergy with TNF-α, regulating the expression of the four 11p15 mucin genes, MUC2, MUC5AC, MUC5B, and MUC6, as well as identify promoter regions involved in that regulation. For that, studies were conducted in the human lung mucoeipidermoid cell line NCI-H292, which has been extensively used until now to study mucin gene regulation in lung cancer cells.
EGF (25 ng/ml) or TGF-α/H9251 and MUC2 in this report that MUC2 is also up-regulated by EGF and work on the regulation of that gene. However, we clearly show described by Nadel and co-workers (9, 10), who had focused their regarding MUC5AC regulation is in agreement with those de-

MUC5AC promoters, respectively. However, ERK-signaling cascade is not the exclusive pathway since expression of MUC2 and MUC5AC mRNAs was not totally abolished after inhibition of ERK by PD98059 or U0126. Because EGFR may also transduce signals through JNK (51), p38 (51), phosphatidylinositol 3-kinase (52), or protein kinase C (51), these signaling cascades will also have to be investigated in the future. Preliminary data in the laboratory suggest that protein kinase C-signaling pathway is also involved in mediating MUC2 and MUC5AC up-regulation by EGFR ligands since partial inhibi-

tion of their expression was observed when NCI-H292 cells were pretreated with protein kinase C inhibitor GF109203X.

Regarding synergism between TNF-α- and EGF-mediated activation of mucin gene expression, previous work carried out in NCI-H292 cells showed that activation of EGFR tyrosine kinase by its ligands (EGF, TGF-α) leads to the synthesis of MUC5AC mRNA and protein via mitogen-activated protein kinase p44/42 MAPK-signaling pathway and that this phenomenon is potentiated by TNF-α (9). We, however, did not find any synergistic effect between TNF-α and EGF or TGF-α, both, at the mRNA level or on promoter activity. Regarding the direct effect of TNF-α on mucin gene expression, previous studies also carried out in NCI-H292 cells have shown that TNF-α up-regulates MUC2 (47) but not MUC5AC (9). In this paper, we show that both genes are up-regulated at the mRNA level by TNF-α, but we could not show any significant up-regulation of MUC2 and MUC5AC promoters. This means that TNF-α-responsive elements are located in other parts of the 5'-flanking regions of the genes and/or post-transcriptional mechanisms like mRNA stabilization are occurring or that an indirect mechanism via activation of EGFR by TNF-α is involved (19).

We then went further into our analysis of MUC2 and MUC5AC regulation at the promoter level by EGF and TGF-α. Recently, it was shown that the up-regulation by EGF of genes that are involved in carcinogenesis often implicates cooperative mechanisms mediated by ubiquitous transcription factor Sp1. The mechanism either involves activation of Sp1 by a specific kinase (53) or uses Sp1 as a docking platform to convey EGF-activated AP-1 transcription factor toward the promoter of the target gene when AP-1 binding sites are absent in the promoter

These cells are known to express EGFR, MUC2, and MUC5AC (9, 10, 46–50). In this report we also show that these cells express MUC5B mRNA but not the protein. Our data quickly showed that MUC5B and MUC6 were not submitted to EGF and TGF-α regulation. On the other hand MUC2 and MUC5AC, the two central genes in the cluster, are up-regulated both at the mRNA and protein levels (Fig. 9). The data regarding MUC5AC regulation is in agreement with those described by Nadel and co-workers (9, 10), who had focused their work on the regulation of that gene. However, we clearly show in this report that MUC2 is also up-regulated by EGF and TGF-α, raising the question whether those two genes contain common regulatory elements in their promoters.

To answer this question, pharmacological agents with specific inhibitory activity were used both in RT-PCR experiments and in functional studies. We have demonstrated that EGF- and TGF-α-mediated up-regulation of MUC2 and MUC5AC goes through binding of EGFR and then activation of the Ras/Raf/ERK-signaling cascade. Responsive elements were localized in the −2627/−2097 and −202/−1 regions of MUC2 and MUC5AC promoters, respectively. However, ERK-signaling cascade is not the exclusive pathway since expression of MUC2 and MUC5AC mRNAs was not totally abolished after inhibition of ERK by PD98059 or U0126. Because EGFR may also transduce signals through JNK (51), p38 (51), phosphatidylinositol 3-kinase (52), or protein kinase C (51), these signaling cascades will also have to be investigated in the future. Preliminary data in the laboratory suggest that protein kinase C-signaling pathway is also involved in mediating MUC2 and MUC5AC up-regulation by EGFR ligands since partial inhibi-
Because Sp1 is an important regulator of MUC2 (12, 17), MUC5AC (this report), and MUC5B (15, 16) expression, we tested its potential involvement in their EGF-mediated up-regulation. Our data clearly indicate that Sp1 is essential to get efficient up-regulation of MUC2 and MUC5AC after EGF and TGF-α stimulation, since inhibition of Sp1 binding to DNA by mithramycin A efficiently inhibited EGF- and TGF-α-mediated up-regulation of these two genes. So far, electrophoretic mobility shift assay studies performed in the laboratory failed to identify AP-1 binding sites in MUC2 and MUC5AC promoters. We, thus, hypothesize that Sp1 may act as a docking platform to help convey the transcription factor(s) activated by EGF or TGF-α.

Transcription factors of the Sp family have recently emerged as potent regulators of mucin genes (18). Having previously shown that MUC5B is regulated not only by Sp1 but also by Sp3 transcription inhibitor (16), we studied its effect on MUC2 and MUC5AC promoters as well. It is of importance to note that when both transcription factors are present in equimolar amounts, Sp3 inhibitory effect prevails and leads to the absence of transactivation of the genes. Sp3 appears thus as a potent inhibitor of 11p15 mucin gene expression. Our results indicate also that Sp1 binding is implicated in MUC2 basal transcriptional activity in NCI-H292 cells since mithramycin A blocks its expression at promoter and mRNA levels. This transcription factor appears important for MUC2 gene expression since it interacts and transactivates murine (17), rat (55), and human (Ref. 17 and this report) MUC2 promoters. This result is of importance because 11p15 mucin genes are clustered in a GC-rich region of chromosome 11 (1, 18), where gene methylation is often associated with their repression in cancer and poor prognosis (16, 18, 32, 42). The relationship between Sp1/Sp3 expression and 11p15 mucin gene methylation status in cancer cells appears as a common regulatory mechanism (18) and will have to be evaluated in pathologies in which repression of mucin genes is observed.

In conclusion, our study performed on the four 11p15 mucin genes show that they are tightly and differentially regulated in human NCI-H292 lung cancer cells. The two central genes of the cluster, MUC2 and MUC5AC, are up-regulated by EGF and TGF-α, whereas MUC6 and MUC5B are not. TGF-α-mediated up-regulation of MUC2 and MUC5AC was shown to be mediated by Sp1, a ubiquitous transcription factor that itself is able to induce transcription of all 11p15 mucin genes. Our studies will certainly help define new therapeutic strategies in airway hypersecretory diseases and lung carcinogenesis in which one may want to efficiently and selectively inhibit the EGF-signaling pathway to reduce/prevent 11p15 mucin hypersecretion and/or 11p15 mucin gene-altered expression.

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FIG. 9. Schematic representation of the signaling pathways leading to MUC2 and MUC5AC up-regulation in response to EGF, TGF-α and TNF-α in NCI-H292 lung cancer cells. Sp1 and Sp3 biological roles on 11p15 mucin gene expression are also indicated. MEK, mitogen-activating protein/ERK kinase.
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