Mucin biosynthesis: upregulation of core 2 β1,6 N-acetylgalactosaminyltransferase by retinoic acid and Th2 cytokines in a human airway epithelial cell line

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Beum, Paul V., Hesham Basma, Dhundy R. Bastola, and Pi-Wan Cheng. Mucin biosynthesis: upregulation of core 2 β1,6 N-acetylgalactosaminyltransferase by retinoic acid and Th2 cytokines in a human airway epithelial cell line. Am J Physiol Lung Cell Mol Physiol 288: L116–L124, 2005; doi:10.1152/ajplung.00370.2003.—Vitamin A and the T helper 2 cytokines IL-4 and IL-13 play important roles in the induction of mucin gene expression and mucus hypersecretion. However, the effects of these agents on enzymes responsible for mucin glycosylation have received little attention. Here, we report the upregulation of core 2 β1,6 N-acetylgalactosaminyltransferase (C2GnT) activity both by all-trans retinoic acid (RA) and by IL-4 and IL-13 in the H292 airway epithelial cell line. Northern blotting analysis showed that the M isoform of C2GnT, which is expressed in mucus-secreting tissues and can form all mucin glycan β1,6-branched structures, including core 2, core 4, and blood group I antigen, was upregulated by both RA and IL-4/13. The L isoform, which forms only the core 2 structure, was moderately upregulated by IL-4/13 but not by RA. Enhancement of the M isoform of C2GnT by RA was abolished by an inhibitor of RA receptor α, implicating RA receptor α in the effect of RA. Likewise, an inhibitor of the Janus kinase 3 pathway blocked the enhancing effects of IL-4/13 on the L and M isoforms of C2GnT, suggesting a role of this pathway in the upregulation of these two C2GnTs by these cytokines. Taken together, the results suggest that IL-4/13 T helper 2 cytokines and RA can alter the activity of enzymes that synthesize branching mucin carbohydrate structure in airway epithelial cells, potentially leading to altered mucin carbohydrate structure and properties.

Mucin carbohydrate; core 2 glycosyltransferases; interleukin-4; interleukin-13; T helper 2

MUCINS ARE HIGH-MOLECULAR-WEIGHT GLYCOPROTEINS produced in the airways, gastrointestinal tract, genitourinary tract, breast, and pancreas that help lubricate these tissues and protect them from dehydration and pathogenic insult (45). To date, at least 19 mucin cDNAs have been cloned (6). Mucins possess a high content of serine and threonine residues, which serve as sites for attachment of mucin-type glycans, the major determinants of mucin function.

Mucin carbohydrate biosynthesis (45) begins with the attachment of N-acetylgalactosamine (GalNAc) to a serine or threonine residue as catalyzed by peptidyl GalNAc transferases (pGalNAc TF). The synthesis of β1,6 branches in the chain, which involves the attachment of N-acetylgalactosamine (GalNAc) in β1,6 linkage to galactose in GalNAcβ1,3-Galβ1,6-R or GalNAc in GalNAcβ1,3-GalNAcα-Ser/Thr, generally leads to larger, more complex chains possessing greater functional potential.

Enzymes exhibiting core 2 β1,6 N-acetylgalactosaminyltransferase (C2GnT) activity catalyze the formation of an early β1,6 branch point during mucin glycan biosynthesis, as shown below:

$$\text{UDP-GlcNAc} + \text{Galβ1,3GalNAcα-Ser/Thr} \rightarrow \text{UDP}$$

Galβ1,3GalNAcα-Ser/Thr

Three distinct enzymes containing C2GnT activity have been reported to date, referred to as leukocyte-type C2GnT (C2GnT-L or C2GnT-1) (5, 26, 29), mucus-type C2GnT (C2GnT-M or C2GnT-2) (8, 25, 40, 42, 60), and thymus-type C2GnT (C2GnT-T or C2GnT-3) (43). These three enzymes are distinguished by nucleotide and amino acid sequences, tissue distribution, and carbohydrate structures they are able to form (3). Both C2GnT-L and C2GnT-3 can create only the core 2 β1,6 GlcNAc branched structure (5, 43), whereas C2GnT-M can additionally synthesize the core 4 structure, GlcNAcβ1-3(GalNAcβ1-6)GalNAcα-Ser/Thr (C4GnT), and the blood group I structure, GlcNAcβ1-3(GalNAcβ1-6)Galβ-R (IGnT) (8, 40, 43, 60), as shown below.

$$\text{UDP-GlcNAc} + \text{GalNAcβ1,3GalNAcα-Ser/Thr} \rightarrow \text{UDP}$$

GlcNAcβ1,3GalNAcα-Ser/Thr

The core 2 β1,6 GlcNAc branch is a structural component of selectins ligands that are implicated in the binding of tumor cells (31, 46, 54) and leukocytes (35, 53) to endothelial cells.
C2GnT activity is often altered in cancer tissues and may contribute to the aberrant expression of tumor-associated antigens on MUC1 in such tissues (4). Expression of MUC5AC gene has been shown to be upregulated during all-trans retinoic acid (RA)-induced differentiation of human bronchial epithelial cells (15, 16, 18, 23, 24, 49, 61). Also, IL-4 and IL-13 have been shown to upregulate MUC5AC expression and secretion of mucous glycoconjugates in mouse airway (12, 44, 48). In addition, IL-13 can suppress MUC5AC expression in primary culture of guinea pig tracheal epithelium (22). However, relatively little is known about the effects of RA and cytokines on the glycosyltransferases that synthesize mucin carbohydrate. We therefore examined the effects of RA and T helper 2 (Th2) cytokines IL-4 and IL-13 on expression of MUC5AC protein and other key mucin glycosyltransferases in the H292 cell line. We found that both RA and IL-4/13 upregulate C2GnT activity, acting primarily on the expression of C2GnT-M isozyme. C2GnT-L was upregulated by IL-4/13 but not by RA. An antagonist of RA receptor α (RARα) mitigated the effects of RA on C2GnT-M, and the effects of IL-4/13 were abolished by treatment with an inhibitor of the Janus kinase (JAK) 3 pathway, thus providing information about the possible mode of action of these agents on C2GnT-L and C4GnT-M.

MATERIALS AND METHODS

Materials. The NCI-H292 cell line was purchased from ATCC (Manassas, VA). CMP-[3H]NeuAc (10–20 Ci/mmol), UDP-[3H]GlcNAc (60 Ci/mmol), UDP-[3H]Gal (60 Ci/mmol), and UDP-[3H]GalNAc (15 Ci/mmol) were obtained from the American Radiolabeled Chemicals (St. Louis, MO). All-trans RA, acetylmethionin D, and cycloheximide were purchased from Sigma (St. Louis, MO). Galβ1-3GalNAc-Bzl (Bzl) GlcNAcβ1–3GalNAcα-p-nitrophenyl were purchased from Toronto Research Chemical (Toronto, Canada). IL-4, IL-5, IL-9, and IL-13 were purchased from Life Technologies (Rocky-ridge, MD), RO41-5253, a specific agonist of RARα (23), was kindly provided by Dr. S. K. Batra, The Nebraska Medical Center, Omaha, NE. Bisindolylmaleimide I, PD98059, AG490, wortmannin, and JAK3 inhibitor II were obtained from Calbiochem (La Jolla, CA). Immobilon P polyvinylidene fluoride membrane was purchased from Millipore (Bedford, MA). Bond Elut C18 cartridges were from Varian (Sunnyvale, CA). Other chemicals were from Sigma (St. Louis, MO).

Cell culture. NCI-H292 cells were routinely grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin). Effector treatments were performed in this same medium, starting at ~75% confluency, using variable concentrations of RA, IL-4, and IL-13, 1 μM RO41-5253, 20 ng/ml IL-5, 20 ng/ml IL-9, 300 nM wortmannin, 50 μg/ml JAK3 inhibitor II, 25 ng/ml PD98059, 5 μM AG490, and 1 μM bisindolylmaleimide I. In experiments involving cotreatment with IL-4 and inhibitors, cells were pretreated for 1 h with inhibitor before addition of IL-4/13.

Glycosyltransferase enzyme assays. Total cell homogenates used in enzyme assays were prepared as follows. Cells in T25 flasks were washed twice with cold PBS, scraped in 200 μl of 0.25 M sucrose, and successively passed through 18-, 20-, and 26-gauge syringe needles to disrupt the cell membrane. Protein concentration was measured by the Bio-Rad assay (Bio-Rad) using BSA as the standard. All enzyme assays were conducted under conditions in which product formation was linear with respect to time and enzyme amount. An additional reaction without exogenous acceptor was performed to measure background activity for reactions with endogenous acceptors. Enzyme activity was calculated by subtracting endogenous activity from total activity and was expressed as nanomoles of sugar transferred per hour per milligram of total protein. C2GnT activity was assayed essentially as described (40), using Galβ1–3GalNAc-Bzl as acceptor. Briefly, the reaction mixture contained 50 mM MOPS, pH 7.5, 5 mM MnCl2, 2% Tween 20, 1 mg/ml BSA, 2 mM UDP-GlcNAc containing 6,400 dpm/μmol UDP-[3H]GlcNAc, and 1 mM ATP in a final volume of 50 μl. Reactions with core 1 acceptor contained 2 mM Galβ1–3GalNAc-Bzl, whereas background reactions contained water instead of acceptor. The reaction mixtures were incubated for 2 h at 37°C, then terminated by the addition of 300 μl of 10 mM ZnCl2. A 10-μl aliquot was measured by scintillation counting for total radioactivity in the reaction mixture, then 300 μl of the mixture was applied to a C18 cartridge under house vacuum. The cartridges were washed nine times with 2.5 ml of 0.1 M Tris(hydroxymethyl)-aminomethane(Tris)-HCl, pH 7.5; then the product was eluted four times with 0.5 ml of methanol each. The methanol was evaporated in a Speed-Vac (Savant Instruments, Holbrook, NY), and the residue was dissolved in 0.5 ml of water and measured for radioactivity by scintillation counting. Core 1 β1,6-N-acetylglucosaminyltransferase (C4GnT) activity was measured identically to C2GnT, except that 2 mM GlcNAcβ1,3GalNAcα-p-nitrophenyl was used as acceptor. pGalNAc TF was assayed as described (40), except that the reaction mixture contained 0.1 mM UDP-GalNAc containing 105 dpm/μmol UDP-[3H]GalNAc, 1 mg/ml BSA, and 0.1 mg/ml acceptor peptide. The acceptor peptide consisted of a synthetic 29-amino-acid CUC2 peptide having the sequence PTTPTTTTTTTVTPTPTGTTPTPTTPTTI.

Northern blotting and RT-PCR. Total RNA was isolated from harvested cells using TRI reagent (MRC, Cincinnati, OH) according to the manufacturer’s protocol. RNA (10–20 μg) was electrophoresed on formaldehyde-agarose gels and then capillary-blotted to a Nytran membrane (Schleicher and Schuell, Keene, NH). The membrane was cross-linked by ultraviolet and treated with 1% SDS and 0.1 M NaCl at room temperature for 1 h. This prehybridization solution was then replaced by 25 ml of hybridization solution HS-114 (MRC) to which ~1 × 107 dpm of denatured [3P]-labeled oligodeoxynucleotide probe against C2GnT-M, C2GnT-L, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) had been added. The probe for C2GnT-M consisted of the sequence from position 383 to 667 in the cDNA sequence (relative to the translation start site) (42, 60), whereas that for C2GnT-L ran from position 423 to 685 in the C2GnT-L cDNA sequence (5). These probes were chosen so as to maximize specificity for the desired C2GnT isoform. The GAPDH probe consisted of an 1800-bp PstI/XbaI fragment from the 5’ end of the human GAPDH (ATCC). Probe hybridization was carried out at 62°C for 24–48 h, followed by washing five times, 10 min each, at 55°C in 1× SSC (0.44% sodium citrate, 0.877% sodium chloride). The membrane was then exposed to autoradiography film overnight. RT-PCR for assessing expression of C2GnT-L, C2GnT-M, and GAPDH was performed using the primers as described above.

Western blotting detection of MUC5AC. Cell lysates (90 μg of protein) from untreated control H292 cells or H292 cells treated with RA or IL-4 were fractionated on a 1% agarose gel containing 0.1% SDS and 0.25 M Tris, pH 8.8, then transferred to polyvinylidene

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Because of the functional importance of mucin carbohydrate expression (data not shown), validating published reports of RA-induced MUC5AC expression in the H292 cell line, consistent with previous studies of the effects of RA on bronchial epithelial cell cultures and tracheal explants. We also performed anti-MUC5AC blots on lysates of H292 cells treated for 24 h with 20 ng/ml IL-13 but saw no apparent effect on MUC5AC protein levels than cells grown in the absence of RA (data not shown). This result suggests that RA is able to induce a more differentiated phenotype in the H292 cell line, consistent with previous studies of the effects of RA on bronchial epithelial cell cultures and tracheal explants. We also performed anti-MUC5AC blots on lysates of H292 cells treated for 24 h with 20 ng/ml IL-13 but saw no apparent effect on MUC5AC expression (data not shown), validating published report (12, 41).

RA and Th2 cytokines upregulate C2GnT activity in H292 cells. Because of the functional importance of mucin carbohydrate, we next examined the effect of RA, IL-4, and IL-13 on the activities of several mucin glycosyltransferases involved in the early steps of mucin carbohydrate biosynthesis. We assayed whole cell extracts for the activity of 1) pGalNAc TF (11), which initiates glycosylation of mucin-type glycans and thus serves as an index for the degree of mucin glycosylation; 2) UDP-Gal: αGalNAc β1–3 Gal TF (core 1 Gal TF), which forms the obligatory acceptor substrate for C2GnT; 3) C2GnT; 4) CMP-NeuAc: Galβ1,3GalNAc (NeuAc to Gal) α2,3 sialyltransferase (ST3 Gal I), which also utilizes the core 1 acceptor substrate, potentially blocking the action of C2GnT on the acceptor (26); and 5) C4GnT, which serves as a measure of the level of C2GnT-M expression, because C2GnT-M is the sole enzyme that exhibits C4GnT activity (3, 8, 25, 40, 42, 60). As shown in Table 1, treatment of H292 cells with 100 nM RA upregulated pGalNAc TF, C2GnT activity, and C4GnT activity. RA also enhanced core 1 Gal TF and ST3 Gal I activities.

**Table 1. Effects of RA and IL-13 on glycosyltransferase activities in H292 cells**

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Untreated 96 h</th>
<th>100 nM RA 96 h</th>
<th>Untreated 24 h</th>
<th>20 ng/ml IL-13 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGalNAc TF Sp. Act. (nmol/h/mg)</td>
<td>1.12±0.41</td>
<td>2.00±0.13*</td>
<td>1.95±0.67</td>
<td>1.45±0.17</td>
</tr>
<tr>
<td>ratio</td>
<td>1.0</td>
<td>1.8</td>
<td>1.0</td>
<td>0.75</td>
</tr>
<tr>
<td>Core 1 Gal TF Sp. Act. (nmol/h/mg)</td>
<td>30.47±3.00</td>
<td>42.90±6.30</td>
<td>40.40±2.50</td>
<td>53.40±11.30</td>
</tr>
<tr>
<td>ratio</td>
<td>1.0</td>
<td>1.4</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>C2GnT Sp. Act. (nmol/h/mg)</td>
<td>9.28±0.46</td>
<td>17.51±0.30†</td>
<td>5.90±0.89</td>
<td>16.21±1.47*</td>
</tr>
<tr>
<td>ratio</td>
<td>1.0</td>
<td>1.9</td>
<td>1.0</td>
<td>2.8</td>
</tr>
<tr>
<td>C4GnT Sp. Act. (nmol/h/mg)</td>
<td>4.04±0.63</td>
<td>7.28±0.46*</td>
<td>1.72±0.65</td>
<td>7.94±1.25*</td>
</tr>
<tr>
<td>ratio</td>
<td>1.0</td>
<td>1.8</td>
<td>1.0</td>
<td>4.5</td>
</tr>
<tr>
<td>ST3 Gal I Sp. Act. (nmol/h/mg)</td>
<td>12.10±2.10</td>
<td>23.20±4.30</td>
<td>30.00±6.50</td>
<td>21.80±8.90</td>
</tr>
<tr>
<td>ratio</td>
<td>1.0</td>
<td>1.9</td>
<td>1.0</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Values are means±SE, n=4 independent T25 flasks. H292 cells were grown for 96 h in the presence or absence of 100 nM retinoic acid (RA), or 24 h in the presence or absence of 20 ng/ml IL-13, and then whole cell extracts were assayed for the activities of the enzymes indicated. Ratio, enzyme activities relative to that of the control. pGalNAc TF: N-acetylglucosaminyltransferase; C2GnT, core 2 β1,6-N-acetylgalactosamine transferase; C2GnT, core 2 β1,4-acetylglucosaminyltransferase; C4GnT, core 4 β1,4-N-acetylglucosaminyltransferase; ST3 Gal I, CMP-sialic acid:Galβ1,3GalNAc (sialic acid-Gal)α2,3 sialyltransferase. Statistically significant difference in means between control and treated samples: *P < 0.01; †P < 0.05; **P < 0.01; ***P < 0.001.

Fluoride membrane at 250 mA overnight. The blot was probed with 1:250 anti-MUC5AC monoclonal antibody (clone 45M1, NeoMarkers, Fremont, CA) in 5% nonfat milk/Tris-buffered saline (TBS), then with 1:2,000 goat anti-mouse (horseradish peroxidase conjugate) secondary antibody in 5% nonfat milk/TBS, and developed using enhanced chemiluminescence (Amersham-Pharmacia Biotech, Arlington Heights, IL).

Statistical analysis of data. The Prism (GraphPad, San Diego, CA) software package was used for computing two-tailed P values using an unpaired t-test.

**RESULTS**

**RA induces expression of MUC5AC in H292 cells.** Vitamin A is a well-known factor involved in the development and differentiation of airway epithelial mucosa (9, 10, 14, 18, 32–34, 58, 61), and all-trans RA induces the expression of specific mucin genes such as MUC2, MUC4, MUC5AC, and MUC5B in airway cell cultures (1, 15, 16, 18, 23, 24, 49, 61). We used a monoclonal antibody directed against MUC5AC to examine whether RA can induce MUC5AC expression in the H292 airway cell line. We found that H292 cells treated with 100 nM RA for 96 h produced significantly more MUC5AC protein levels than cells grown in the absence of RA (data not shown). This result suggests that RA is able to induce a more differentiated phenotype in the H292 cell line, consistent with previous studies of the effects of RA on bronchial epithelial cell cultures and tracheal explants. We also performed anti-MUC5AC blots on lysates of H292 cells treated for 24 h with 20 ng/ml IL-4 but saw no apparent effect on MUC5AC expression (data not shown), validating published report (12, 41).

**RA and Th2 cytokines upregulate C2GnT activity in H292 cells.** Because of the functional importance of mucin carbohydrate, we next examined the effect of RA, IL-4, and IL-13 on the activities of several mucin glycosyltransferases involved in the early steps of mucin carbohydrate biosynthesis. We assayed whole cell extracts for the activity of 1) pGalNAc TF (11), which initiates glycosylation of mucin-type glycans and thus serves as an index for the degree of mucin glycosylation; 2) UDP-Gal: αGalNAc β1–3 Gal TF (core 1 Gal TF), which forms the obligatory acceptor substrate for C2GnT; 3) C2GnT; 4) CMP-NeuAc: Galβ1,3GalNAc (NeuAc to Gal) α2,3 sialyltransferase (ST3 Gal I), which also utilizes the core 1 acceptor substrate, potentially blocking the action of C2GnT on the acceptor (26); and 5) C4GnT, which serves as a measure of the level of C2GnT-M expression, because C2GnT-M is the sole enzyme that exhibits C4GnT activity (3, 8, 25, 40, 42, 60). As shown in Table 1, treatment of H292 cells with 100 nM RA upregulated pGalNAc TF, C2GnT activity, and C4GnT activity. RA also enhanced core 1 Gal TF and ST3 Gal I activities.

**Fig. 1.** Retinoic acid (RA) and IL-13 upregulate N-acetylgalactosaminyltransferase (C2GnT) activity in a time- and dose-dependent fashion. H292 cells were measured for C2GnT specific activity (Sp. Act.) after being cultured for 24 h in the absence or presence of the indicated concentrations of IL-13 (B), and for varying lengths of time in the presence of 100 nM RA (C) or 20 ng/ml IL-13 (D). *P < 0.05; **P < 0.01; ***P < 0.001. Error bars show SE; n = 4 independent T25 flasks.
although the effects were not statistically significant. IL-13 also significantly enhanced C2GnT and C4GnT activities and upregulated core 1 Gal TF activity by 32%. However, IL-13 lowered pGalNAc TF and ST3 Gal I activities by ~25–27%. A separate experiment showed that 24 h of treatment with 20 ng/ml IL-4 enhanced C2GnT activity from 6.01 ± 1.66 to 14.1 ± 1.44 nmol·h⁻¹·mg protein⁻¹, an effect similar to that of IL-13.

The consistent enhancement of C2GnT and C4GnT activities by RA and IL-4/13 prompted us to further characterize the effects of these agents on enzymes that exhibit these activities. Figure 1 showed that the enhancement of C2GnT activity by RA or IL-13 exhibited a dose and time dependency. It takes 72 h for RA but only 12 h for IL-13 to produce a significant effect. The minimal doses for RA and IL-13 to induce a significant change are 20 nM and 10 ng/ml, respectively. Cotreatment of H292 cells with IL-4 and IL-13 did not lead to further enhancement of C2GnT activity beyond that caused by IL-4 alone (Fig. 2A). Also, C4GnT activity was not stimulated further by IL-4 and IL-13 combination treatment (data not shown). We also treated H292 cells with IL-5 and IL-9, two additional Th2 cytokines known to contribute to asthmatic airway inflammation (27, 30, 48, 53). We saw no significant effect on C2GnT activity (data not shown), despite the fact that H292 cells are known to possess functional IL-5 and IL-9 receptors (28). Interestingly, cotreatment of H292 cells with RA and IL-4 led to an enhancement of C4GnT activity (26.2 nmol·h⁻¹·mg protein⁻¹) greater than the sum (14.2 nmol·h⁻¹·mg protein⁻¹) of the activities enhanced by either agent, i.e., 9.9 and 4.3 nmol·h⁻¹·mg protein⁻¹ for IL-4 and RA, respectively (Fig. 2C).

RA and IL-4/13 selectively enhance C2GnT-M expression.

To date, three glycosyltransferases have been identified that exhibit C2GnT activity (C2GnT-L, C2GnT-M, and C2GnT-3 (3)), and thus the results of the C2GnT assay shown in Table 1 potentially reflect contributions from one or several of these enzymes. As described above and shown in Table 1, RA and IL-13 treatments resulted in a substantial enhancement of C2GnT and C4GnT activities, suggesting that C2GnT-M is at least partially responsible for the overall enhancement of C2GnT activity caused by these agents since C2GnT-M is the lone enzyme known to exhibit C4GnT activity (3). We next performed Northern blotting analysis using probes specific for C2GnT-L and C2GnT-M. Blots on RNA isolated from H292 cells treated for 96 h with increasing concentrations of RA revealed that C2GnT-M was upregulated in a dose-dependent manner by RA (Fig. 3A), mirroring the dose-dependent effect of RA on C2GnT activity (Fig. 1C). However, RA did not affect the C2GnT-L mRNA level.

C2GnT-M mRNA level was upregulated on treatment with 20 ng/ml IL-13 for increasing periods of time (Fig. 3B), consistent with the time-dependent increase in C2GnT activity caused by IL-13 (Fig. 1B). Contrary to RA, which did not affect C2GnT-L mRNA expression (Fig. 3A), IL-13 moderately enhanced C2GnT-L mRNA level (Fig. 3B). Similar to IL-13, IL-4 also enhanced the expression of both C2GnT-M and C2GnT-L mRNAs with a greater enhancing effect on C2GnT-M (Fig. 2B). IL-4 and IL-13 combination treatment did not further increase the effect beyond that induced by IL-4 treatment (Fig. 2B).

To address the question of whether IL-4/13-induced enhancement of C2GnT activity was the result of increased transcription and/or translation, we measured C2GnT activity in H292 cells treated with transcription inhibitor actinomycin D and translation inhibitor cycloheximide. Both agents were found to block the ability of IL-4/13 to enhance the expression of not only enzyme activities of C2GnT and C4GnT (Fig. 4, A and B) but also mRNAs of C2GnT-L and C2GnT-M (Fig. 4C).

Fig. 2. Effects of co-treatment of H292 cells with IL-4 and IL-13 or IL-4 and RA on C2GnT and C4GnT activities. A: C2GnT activity in H292 cells grown 24 h in the absence or presence of 20 ng/ml IL-4 alone or in combination with 20 ng/ml IL-13. B: Northern blots of total RNAs isolated from cells were probed for C2GnT-M, C2GnT-L, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; as internal control). The relative ratios of the bands to that of the control are listed at the bottom of each band. C: H292 cells grown for 24 h in the presence of 20 ng/ml IL-4, 500 nM RA, or both. Whole cell extracts were then assayed for C4GnT activity. P values for treated vs. control samples are **P < 0.05 and ***P < 0.001. Error bars show SE; n = 4 independent T25 flasks.
These results were obtained by Western blotting (Fig. 4C). These agents also blocked the enhancing effects of IL-4 (data not shown).

Potential signaling pathways involved in the enhancement of C2GnT activity by RA and IL-4/13. We examined the mechanism of action of RA and IL-4/13 on C2GnT with inhibitors of specific signaling pathways. Cotreatment of H292 cells with RA and RO41-5253, a specific inhibitor of RA binding to RARα, resulted in ablation of the RA-mediated enhancement of both C2GnT and C4GnT activities (Fig. 5, A and B), suggesting involvement of RARα in the RA action on C2GnT-M.

To identify the active pathway by which IL-4/13 stimulates C2GnT and C4GnT activities, we used specific inhibitors to block JAK2-activated pathways (AG490), JAK3-activated pathways (JAK3 inhibitor II) (57), the Ras-MAP kinase pathway (PD98059), and the phosphatidylinositol 3-kinase-protein kinase C pathway (wortmannin and bisindolylmaleimide I). We first pretreated the cells with these inhibitors, then added IL-4/13 to a final concentration of 20 ng/ml, and incubated the cells for 24 h. Because both IL-4 and IL-13 gave similar results, only the results obtained with IL-13 are given. JAK3...
zymes that catalyze mucin carbohydrate biosynthesis. One of the issues we addressed in this report is whether synthesis of mucin and mucin carbohydrate is coordinately regulated. We examined the effects of RA and IL-4/13, which are known modulators of mucin gene expression and airway mucin production (15, 16, 18, 23, 24, 32, 49, 61), on the expression of MUC5AC and the activities of several important mucin glycosyltransferases in the H292 airway epithelial cell line. We found that RA upregulated MUC5AC and the activities of several key glycosyltransferases, including pGalNAC TF, core 1 Gal transferase, C2GnT-M, and ST3Gal I enzymes. These enzymes are needed for the synthesis of mature mucins to produce secretory granules, the mucus cell differentiation marker (15, 16, 18, 23, 24, 49, 61). Our results are consistent with the mucus cell differentiation property of RA. Selective upregulation of C2GnT-M mRNA level without affecting C2GnT-L mRNA level by RA indicates an association of C2GnT-M but a lack of association of C2GnT-L with mucus cell differentiation. It is known that the expression of C2GnT-L is not restricted to mucus-secreting tissues. However, there was a long lag time, i.e., >48 h, in the upregulation of both C2GnT-M mRNA and enzyme activity (Fig. 1, A and C). The results suggest that the RA-induced upregulation of C2GnT-M is the result of RA action indirectly through RARα-regulated pathways. We also found that, in H292 cells, the RA-mediated upregulation of C2GnT activity did not result in an apparent increase in the structural complexity of mucin carbohydrate on FLAG epitope-tagged MUC1 (data not shown). High basal C2GnT activity in H292 cells may explain why enhancement of C2GnT activity by RA did not affect the complexity of mucin carbohydrate. However, we cannot rule out the possibility that other mucins in the H292 cells might show increased core 2 branching in response to RA treatment because we only examined the carbohydrate structure of MUC1.

Similar to RA, IL-4 and IL-13 also increased C2GnT activity in H292 cells (Table 1 and Figs. 1–3). Because C2GnT-M was affected to a greater extent than C2GnT-L, and H292 cells do not express a detectable amount of C2GnT-3 (2), we conclude that the increase in C2GnT activity induced by IL-13 treatment can be attributed primarily to the enhancement of C2GnT-M expression. It is worth noting that IL-4/13 did not increase MUC5AC in H292 cells, which confirmed published reports (12, 41). The result indicates that IL-4 and -13 are not mucus cell differentiation agents. Although the biological significance of IL-4/13-induced upregulation of C2GnT-M in H292 cells is not clear, the moderate enhancement of C2GnT-L may have some biological relevance. C2GnT-L is known to play an important role in immune functions (13, 52). For example, ablation of the C2GnT-L gene in mice resulted in the loss of core 2 structure, which serves as the initiation point from which the essential component, i.e., sialyl Lewis X, of P-, E-, and L-selectin ligands is built (13, 51, 52). As a result, the inflammatory response to injury is compromised (13). It is known that upregulation of C2GnT-L accompanies the activation of T lymphocytes (51, 52), and ectopic expression of C2GnT-L can lead to the formation of core 2-associated sialyl Lewis X (4). Also, sustained expression of C2GnT-L, as demonstrated in transgenic mice (51) and Wiskott-Aldrich syndrome patients (52), can lead to defective T- and B-cell functions. In the case of airway epithelial cells, enhancement of C2GnT activity in airway cells may result in elevated production of core 2-associated sialyl Lewis X on membrane-bound
mucins (4), which may enable epithelial cells to participate in cellular immune defense. The role of mucin glycans in pulmonary immune function is an area that remains to be explored.

IL-4 and IL-13 Th2 cytokines perform their biological functions by binding to the IL-4 receptor $\alpha$, which triggers heterodimerization of this receptor with another protein, such as common gamma C or IL-13 receptor $\epsilon_1$, to form type I or type II receptor, respectively, in a cytokine- and cell type-specific manner (20, 59). IL-4 activates type I and type II receptors, whereas IL-13 activates only the type II receptor in the cells of hematopoietic origin (20, 37, 38, 44). In nonhematopoietic cells, which lack common gamma C (19, 36, 56), both IL-4 and IL-13 can activate type II receptor. In addition, IL-13, not IL-4, can bind IL-13$\alpha_2$ to inhibit type II receptor-mediated signals after internalization (19). Sharing the receptor and signaling pathways, IL-4 and IL-13 have overlapping biological functions (17, 20, 38, 42, 59). However, it is known that IL-13 plays a more prominent effector role in asthma than IL-4 (59).

In addition, IL-4 and IL-13 have an additive effect in pulmonary granuloma formation and airway hyperresponsiveness (59). Because IL-4 and IL-13 combination treatment did not yield a more enhancing effect on C2GnT activity and C2GnT-L and C2GnT-M expression than either one alone, these two cytokines probably employ the same receptor and signaling pathway for their mode of action on these two enzymes. The two known IL-4 and IL-13 signaling pathways are the JAK-STAT and the phosphoinositol 3-kinase insulin responsive sequence-1/2 pathways (59). Our results suggest that the JAK-STAT pathway is involved because inhibition of JAK3 abolishes the effect. This signaling pathway is probably different from that employed by RA, although a cross talk may exist between these two signaling pathways because the IL-4 and RA combination treatment exerted a synergistic effect on C4GnT activity.

Failure of IL-4/13 to stimulate MUC5AC production in vitro, combined with the reported ability of these cytokines to induce mucus cell metaplasia in mouse lung (12, 48), suggests that the in vivo induction of mucus cell metaplasia proceeds through secondary mediators not present in vitro. On the other hand, C2GnT-L and C2GnT-M enzyme activities and messages were upregulated in airway epithelial cells by IL-4 and IL-13. Because these enzyme activities corresponded to transcript levels, higher enzyme activities likely reflect higher transcript levels of these two enzymes rather than increased stability of protein or activation of enzyme activity by post-translational modification. Therefore, increased mRNA levels may represent increased transcription or enhanced mRNA stability. The complete reversal of the IL-13-enhanced C2GnT-L and C2GnT-M mRNA levels by actinomycin D (Fig. 4C) suggests that IL-13 enhances the transcription of these two genes. How IL-13 enhances the transcription of these two genes remains to be investigated.

Recently, we found that EGF selectively downregulated C2GnT-M expression, without significantly affecting C2GnT-L expression (2), and upregulated MUC5AC. These findings, in combination with the selective upregulation of C2GnT-M by RA and IL-4/13 and the differential effects of these agents on MUC5AC in H292 cells, suggest that the H292 cell line is a good model system for studying the regulation of C2GnT-M and MUC5AC by modulators of mucin gene expression and mucus production. These studies could provide a valuable foundation for studying the effects of this regulation on airway epithelial cells in primary culture and in vivo. However, it should be pointed out that H292 cells are immortalized carcinoma cells, which are different from airway epithelial cells in primary culture or in vivo. These cells have a finite life span. Therefore, the observation made in H292 cells should be further confirmed in primary cultures and animals before a conclusion can be made regarding the suitability of using H292 cells as a model system for addressing a biologically relevant question. Furthermore, because modulation of C2GnT-M gene expression in vivo could in principle lead to altered mucin carbohydrate structure and physiological properties of mucins, a better understanding of the regulation of C2GnT-M gene expression is imperative to develop improved therapeutic intervention for patients with mucous hypersecretory disorders.

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REFERENCES


