Expression and regulation of γ-glutamyl transpeptidase-related enzyme in tracheal cells

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Potdar, Pravin D., Kaya L. Andrews, Paul Nettesheim, and Lawrence E. Ostrowski. Expression and regulation of γ-glutamyl transpeptidase-related enzyme in tracheal cells. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L1082–L1089, 1997.—Glutathione plays an essential role in protecting the pulmonary system from toxic insults. γ-Glutamyl transpeptidase-related enzyme (GGT-rel) is a novel protein capable of cleaving the γ-glutamyl peptide bond of glutathione and of converting leukotriene C₄ to leukotriene D₄. A rat homologue of GGT-rel was identified and was found to be highly expressed in cultures of differentiating rat tracheal epithelial (RTE) cells. The 2.6-kb cDNA predicts a 572-amino acid protein with 79% identity to human GGT-rel. GGT-rel was weakly expressed in normal trachea but was strongly induced by epidermal growth factor in cultures of RTE cells. GGT-rel was also highly expressed in lung tumors induced by inhalation of isobutyl nitrite. These results demonstrate that GGT-rel 1) is expressed in normal tracheal cells, 2) can be induced by epidermal growth factor, and 3) is elevated after chemical exposure. The induction of high levels of GGT-rel may play an important role in protecting the lung from oxidative stress or other toxic insults.

The role of GSH and the enzymes involved in GSH metabolism have been the focus of several recent studies of pulmonary cells. For example, exogenous GSH has been shown to protect cultured bovine pulmonary artery endothelial cells from menadione toxicity (3), and oxidative stress increases γ-glutamylcysteine synthetase and GSH (23). In alveolar epithelial cells, oxidative stress increases GGT transcription and activity (15), and increased levels of GGT and γ-glutamylcysteine synthetase have been shown to provide increased resistance to oxidative stress (18). In addition, increased GSH levels may be part of an adaptive response to long-term ozone exposure (8). Glutathione S-transferases (GSTs), which catalyze the addition of the thiol group of GSH to many xenobiotics, may also protect the airways from toxic compounds. A lack of the GST-M1 isoenzyme has been associated with a higher risk of lung cancer (7). Thus GSH and the enzymes responsible for its synthesis and metabolism are of significant importance to the protective mechanisms of the airways in response to a variety of chemical insults.

In our laboratory, we have been investigating the regulation of mucociliary differentiation of rat tracheal epithelial (RTE) cells using a model system in which primary RTE cells undergo many of the processes that occur during injury and repair (14). We have previously shown that the pathway of differentiation can be altered by changing the concentrations of growth factors and hormones used to culture RTE cells. For example, epidermal growth factor (EGF) stimulates mucous cell differentiation and mucus production (11), whereas the removal of EGF increases ciliated cell differentiation (5).

In this work, differential display was used to identify genes for which expression was altered during differentiation of RTE cells under specific conditions. We have identified and cloned the rat homologue of GGT-rel and have demonstrated that its expression can be strongly upregulated by EGF. Additionally, GGT-rel expression was observed to be elevated in lung adenomas and carcinomas from rats chronically exposed to isobutyl nitrite. The increased levels of GGT-rel expression suggest that GGT-rel plays an important role in the metabolism of γ-glutamyl compounds in the airways after injury. Induction of GGT-rel expression may be a protective mechanism in the airways after exposure to toxic insults.

MATERIALS AND METHODS

Materials. Oligonucleotides were obtained from Genosys (The Woodlands, TX) or Research Genetics (Huntsville, AL). All radioactive materials were obtained from Amersham...
(Arlington Heights, IL) except Sequitide, which was obtained from NEN (Boston, MA). All amplifications were performed in a GeneAmp polymerase chain reaction (PCR) system 2400 or 9600 (Perkin-Elmer, Branchburg, NJ). All tissue culture media and reagents were obtained from Sigma Chemical (St. Louis, MO) with the exception of EGF and rat tail collagen, which were purchased from Collaborative Biomedical Products (Bedford, MA), and bovine cell putitaries, which were purchased from Pel-Freeze (Rogers, AR).

Cell culture. Primary RTE cells were cultured using the method originally described by Kaartinen et al. (14) with modifications described in detail previously (22). Briefly, tracheas were obtained from 10- to 14-wk-old male Fischer 344 rats, filled with 1% Pronase, and incubated overnight at 4°C. Primary RTE cells were plated at a density of 2.4 × 10^4 cells/cm^2 on collagen-coated Transwell clear membranes (Costar, Cambridge, MA; see Ref. 2). For all experiments reported here, the cells were submersed in growth factor-supplemented Dulbecco’s modified Eagle’s medium + F-12 medium (complete medium (CM)) until they reached confluency (day 8). On day 8, parallel cultures were divided into the different treatment groups as described for the individual experiments. Cultures were refed daily beginning on day 5. Previous studies have demonstrated that, under these conditions, RTE cells grow predominantly as a pseudostratified mucociliary epithelium with no obvious fibroblast contamination (5, 11, 14). Recent studies from our laboratory have shown that submergence of RTE cells in CM (SUB cultures) inhibits ciliogenesis (21), whereas withdrawal of EGF and cholera toxin (CT) from the media (−EGF/−CT) promotes ciliated cell differentiation (5). Cells were harvested for RNA at different time points, according to the experimental design.

RNA isolation. Cultured cells were collected by scraping with a rubber policeman directly into guanidinium thiocyanate solution and processed for RNA isolation by the method of Chomczynski and Sacchi (4). The same procedure was used to isolate RNA from rat tissues, except the frozen tissues were ground to a powder in liquid N_2, then added to the guanidinium thiocyanate solution. RNA from tracheal epithelium was obtained by simply flushing excised tracheas with the guanidinium thiocyanate solution. RNA from tracheal epithelium was isolated in QuikHyb hybridization solution (Stratagene), according to the manufacturer’s instructions. The amplified cDNAs were then transferred to Nytran membranes (Schleicher & Schuell, Keene, NH) by capillary blotting overnight with 10× SSPE buffer. RNA was cross-linked to the membrane with a stratalinker (Stratagene) and then was prehybridized and hybridized in QuikHyb hybridization solution (Stratagene), according to the manufacturer’s instructions. Nonspecifically bound radioactivity was removed by washing the membrane in 2× standard sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) solutions for 15 min at 70°C. The blots were exposed to Kodak film at −70°C with intensifying screens. For estimation of changes in the expression level of GGT and GGT-rel, Northern blots were analyzed using the Wizard Plus Miniprep DNA Purification System (Promega, Madison, WI) and was sequenced using a Sequenase Kit (Amersham) or an ABI PRISM 377 DNA sequencer and Dye Terminator Cycle Sequencing Kit (Perkin-Elmer).

Sequence analysis. Plasmid DNA was isolated using the Wizard Plus Miniprep DNA Purification System (Promega, Madison, WI) and was sequenced using a Sequenase Kit (Amersham) or an ABI PRISM 377 DNA sequencer and Dye Terminator Cycle Sequencing Kit (Perkin-Elmer). The final sequence was analyzed using the Genetic Computer Group (GCG) sequence-analysis package (version 8; GCG, Madison, WI). Sequences were also analyzed using the MacVector and AssemblyLIGN programs (International Biotechnologies, New Haven, CT).

Northern analysis. Northern analysis was performed using standard procedures essentially as previously described (20). Ten to twenty micrograms of RNA was electrophoresed through a 1.2% agarose-0.75% formaldehyde gel. Ethidium bromide staining demonstrated equal loading before transfer to the membrane. Gels were immersed in 50 mM NaOH for 15 min and were washed twice in 10× sodium chloride/sodium phosphate buffer (SSPE) for 15 min, and RNA was transferred to Nytran membranes (Schleicher & Schuell, Keene, NH) by capillary blotting overnight with 10× SSPE buffer. RNA was cross-linked to the membrane with a stratalinker (Stratagene) and then was prehybridized and hybridized in QuikHyb hybridization solution (Stratagene), according to the manufacturer’s instructions. Nonspecifically bound radioactivity was removed by washing the membrane in 2× standard sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) solutions for 15 min at 70°C with intensifying screens. For estimation of changes in the expression level of GGT and GGT-rel, Northern blots were analyzed using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). To avoid any cross-hybridization between GGT and GGT-rel, duplicate Northern blots were prepared and probed separately for each gene. Blots were also probed with a β-actin cDNA. Results for GGT and GGT-rel were then normalized to the expression level of β-actin as a control for loading and transfer. For the results shown, two completely independent RT-PCR reactions were run in parallel with reproducible results.

Labeling of probes. A rat GGT probe was prepared by RT-PCR of RNA isolated from RTE cell cultures with primers specific for rat GGT. The upstream primer was 5′-AGCCAGG-
TAAGCAACCGCTTTTCGGAATCTGAG-3' (bases 1319–1679 of rat GGT). An annealing temperature of 58°C was used. The 361-bp product was cloned and was verified by sequencing. Probes for GGT-rel and GGT were generated by digestion of plasmid DNA to remove the insert. The cDNA insert was isolated from a low melting point agarose gel, and 25 ng of insert DNA were labeled with the Rediprime DNA Labeling System (Amersham). Probes were purified on NucTrap columns (Stratagene).

RT-PCR analysis. One microgram of RNA from rat brain, heart, kidney, liver, lung, spleen, testis, trachea, or cultured RTE cells was reverse transcribed and amplified with an RNA PCR kit (Perkin Elmer). After reverse transcription, the cDNA samples were amplified using specific primers for rat GGT (as above) or GGT-rel. Primers used for rat GGT-rel were 5'-TGAAGGGAGGGTGAACGTGTAC-3' (upstream) and 5'-TCAATCTGCTGGCGGATGTGCT-3' (downstream). Typical PCR cycling conditions were 95°C for 1 min followed by 35–45 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s, followed by 72°C for 7 min. An aliquot of each reaction was electrophoresed through a 3% NuSieve-Seakem GTC agarose gel and was visualized by ethidium bromide staining.

RESULTS

Isolation of a differentially expressed cDNA during RTE cell differentiation. To isolate genes for which the expression changes during mucous or ciliated cell differentiation, differential display was performed using RNA isolated from RTE cells cultured under three different conditions (described in MATERIALS AND METHODS) that have previously been shown to influence the extent of mucous and ciliated cell differentiation (5, 11, 21). Samples from two independent cultures (days 14–16) were analyzed in parallel. Overall, the differential display banding pattern was highly reproducible between the cultures and between these three conditions (Fig. 1A). With the use of various primer combinations, several differentially expressed bands were identified. One cDNA, which was abundantly expressed in RTE cells cultured in CM at an ALI and in SUB cultures but was not expressed in RTE cells cultured at an ALI in −EGF/−CT (Fig. 1A), was chosen for further characterization in this study.

The cDNA in this band was isolated, reamplified, and used as a probe on Northern blots. The probe detected an abundant transcript of ~2.8 kb in CM and SUB cultures fed with complete growth medium throughout the culture period (Fig. 1B). However, in −EGF/−CT cultures, this message was weakly expressed on day 14 and was not detectable by day 16 (Fig. 1B). This result confirms the differentially expressed pattern observed by differential display.

Cloning and sequencing of rat GGT-rel cDNA. The reamplified cDNA was cloned, and several independent clones were sequenced. The sequence data indicated that during the differential display procedure the cDNA was primed with the arbitrary primer (P3) at both ends. A search of GenBank revealed a high degree of homology between the cloned 373-bp cDNA fragment and human GGT-rel (amino acids 247–270). No significant homology with any other gene was found. To determine the full-length sequence for rat GGT-rel, primers were designed based on the partial sequence, and 5'- and 3'-RACE was performed using a rat brain cDNA library. Several clones for both the 5'- and 3'-ends were obtained and sequenced. The composite 2.6-kb cDNA contained a 1,716-nucleotide open reading frame and codes for a predicted protein of 61,600 kDa. An alignment of the predicted rat and human GGT-rel protein sequences (Fig. 2) demonstrated 79% identity and 82%
similarity. For comparison, an alignment of rat and human GGT also showed 79% identity (83% similarity). Rat GGT-rel contains eight potential N-linked glycosylation sites (Fig. 2), four of which are conserved between the rat and human protein. Rat GGT-rel also contains a two-amino acid insertion (positions 533 and 534) and a 16-amino acid deletion (after position 443) compared with human GGT-rel. Overall, these results indicate that the differentially expressed cDNA is the rat homologue of GGT-rel.

Expression of GGT-rel in rat tissues. To examine the expression of GGT-rel in different tissues, cDNA was
used to probe a Northern blot of RNA isolated from rat tissues and RTE cell cultures. As expected, RNA from RTE cells cultured in CM demonstrated a strong hybridization signal, whereas RNA isolated from tracheal lumen showed only a faint hybridization signal. All other tissues were negative by this method. A faint signal was also observed in RNA isolated from tracheal epithelium by flushing the lumen with lysis solution. However, no message was detected by Northern analysis in all other tissues (brain, heart, kidney, liver, lung, spleen, and testis) tested. To examine the expression of GGT-rel at a more sensitive level, the same tissues were analyzed by RT-PCR using primers specific for GGT-rel. After 45 cycles of amplification using the GGT-rel specific primers, a clear product was visible in all tissues tested (brain, heart, kidney, liver, lung, spleen, testis, and trachea; Fig. 3B). Amplifications performed without RT or without RNA template were routinely negative, indicating the product obtained was dependent on the presence of GGT-rel mRNA. These results indicate that GGT-rel is expressed in many tissues of the adult rat, although at low levels.

Effect of EGF and CT depletion on GGT-rel expression. As shown above, GGT-rel is strongly expressed in cultures grown in CM, but was not detected in cells cultured 7 days in the absence of EGF and CT. To examine the regulation of GGT-rel by EGF and CT, RTE cells were cultured for 7 days in CM, media without EGF, media without CT, or media without EGF and CT. Northern analysis showed that removal of CT from the media had no effect on the expression of GGT-rel (Fig. 4A), whereas the removal of EGF alone reduced GGT-rel expression substantially (72% reduction when normalized to the expression of β-actin). The removal of both EGF and CT caused a further decrease in the expression of GGT-rel. In contrast, the removal of EGF or EGF and CT only slightly reduced GGT expression (−EGF, 36% reduction; −EGF/−CT, 30% reduction; Fig. 4B). These results indicate that EGF has a major effect on the expression of GGT-rel in RTE cells.

Expression of GGT-rel during differentiation in the presence and absence of EGF. To examine the level of expression of GGT-rel during mucociliary differentiation and its regulation by EGF, RTE cells were cultured in media with or without EGF for 7 days after the formation of the ALI. RNA was isolated from parallel
cultures at daily intervals and was analyzed for the level of GGT-rel, GGT, and β-actin transcripts by Northern blotting (Fig. 5, A and B). Removal of EGF from the media resulted in a substantial decrease in the level of GGT-rel expression. When normalized to β-actin expression and compared with RTE cells cultured in CM, RTE cells cultured in the absence of EGF showed a 57% reduction in GGT-rel expression by day 13 (Fig. 5C). By day 15, the level of GGT-rel expression in cultures grown in the absence of EGF was 3.9-fold less than in the CM cultures. The level of GGT expression also decreased after the removal of EGF, although the magnitude of the reduction was <50% on day 15 (Fig. 5C). These results show that GGT-rel is expressed throughout differentiation of RTE cells in vitro and that the removal of EGF causes a substantial reduction in the expression of GGT-rel.

**Induction of GGT-rel by EGF.** To determine if EGF could induce GGT-rel expression directly, RTE cells were grown in media without EGF from days 8 to 13. On days 13 and 14, some of the EGF-depleted cultures were refed with media containing 25 ng/ml EGF. Cells were harvested from the different groups 8, 24, and 48 h after the addition of EGF to the EGF-depleted cultures. Northern analysis demonstrated a 2.4-fold increase (average of 2 experiments) in GGT-rel expression 24 h after addition of EGF (Fig. 6A). This induction was maintained for at least 48 h (2.2-fold, average of 2 experiments), although the level of GGT-rel expression in cultures treated with EGF did not reach the level in parallel cultures grown continuously in CM. GGT expression was also increased after addition of EGF, although not as strongly (1.7-fold at 24 h and 1.4-fold at 48 h, average of 2 experiments; Fig. 6B). EGF is
high levels in lung tissue and tumors from animals, particularly in one of the carcinomas (Fig. 7, lane 5). These results show that GGT-rel is expressed at high levels in lung tissue and tumors from animals chronically exposed to isobutyl nitrite.

DISCUSSION

Using differential display to analyze changes in gene expression during mucociliary differentiation of RTE cells, we have identified a rat homologue of human GGT-rel. This is the first evidence that GGT-rel is expressed in airway epithelial cells. Heisterkamp et al. (13) were unable to demonstrate GGT-rel expression in any of the mouse tissues they analyzed and only observed a very low level of expression in two human cancer cell lines. In contrast, our studies have demonstrated a high level of GGT-rel expression by normal RTE cells in primary culture. In agreement with the results of Heisterkamp et al. (13), GGT-rel expression was not detected by Northern analysis using whole lung or other rat tissues as a source of RNA. However, GGT-rel expression was detectable by RT-PCR in all rat tissues examined, including brain, heart, kidney, liver, lung, spleen, testis, and trachea. This suggests that, similarly to GGT, GGT-rel is widely distributed, although constitutively expressed at much lower levels. Alternatively, it is possible that only a small number of specialized cells express GGT-rel in any particular tissue or that GGT-rel is only highly expressed under certain conditions. In support of the latter hypothesis, our results show that GGT-rel expression can be induced by EGF. Treatment of EGF-deprived RTE cultures with 25 ng/ml of EGF caused a rapid and sustained increase in GGT-rel expression. This demonstrates that GGT-rel expression can be regulated by growth factors and may link the expression of GGT-rel to increased levels of proliferation. In addition, high levels of GGT-rel expression were observed in lung carcinomas, adenomas, and normal lung tissue from rats exposed to isobutyl nitrite, a chemical that causes hyperplasia of the respiratory airway epithelium. Increased levels of GGT expression have been observed in many tumor types, and its expression is frequently used as an early marker for hepatocarcinogenesis (12). Whether GGT-rel expression is induced in tumors as a result of chemical exposure or as a direct result of the neoplastic process is unclear. Additional experiments will be needed to elucidate the possible roles of GGT-rel in these conditions.

GGT-rel has many potential functions in airway epithelial cells. For example, GGT-rel could function in the defense mechanisms of the airways by supplying reducing equivalents to the cell via GSH. Forman and co-workers (3, 15, 18, 23) have demonstrated that GSH can protect pulmonary cells from oxidative stress and that oxidative stress can induce GGT and γ-glutamylcysteine synthetase. Because GGT-rel has been shown to catalyze some of the same reactions as GGT (13), high levels of GGT-rel could also provide protection from oxidative stress. GGT-rel may also play a role in the secretory functions of RTE cells. RTE cell cultures grown in media containing EGF show a high percentage of mucous cells and actively produce and secrete mucus and other products. The withdrawal of EGF decreases mucus production and also reduces the level of GGT-rel expression. Thus GGT-rel may play a role in the secretory pathway, perhaps by providing essential precursors for the synthesis of secreted molecules. In addition, GGT-rel could function in the metabolism of important biological mediators. Human GGT-rel was shown to catalyze the conversion of leukotriene C4 into leukotriene D4, and leukotrienes can cause bronchoconstriction and changes in vascular permeability in the lung (10). At present, the physiological function(s) of GGT-rel in the airways is unknown, but the present work indicates that further studies are warranted.
In summary, the experiments presented here have identified a rat homologue of GGT-rel and demonstrated that GGT-rel is expressed in a wide variety of rat tissues. GGT-rel was shown to be inducible to high levels in cultures of primary RTE cells by EGF. High levels of GGT-rel expression were also observed in several lung tumors and in lung tissue chronically exposed to a carcinogen. Under these conditions, GGT-rel, in addition to GGT, may be important to the metabolism of γ-glutamyl compounds, and future studies will need to distinguish between these two enzymes.

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REFERENCES


