Regulator of G Signaling 16 Is a Marker for the Distinct Endoplasmic Reticulum Stress State Associated with Aggregated Mutant $\alpha_1$-Antitrypsin Z in the Classical Form of $\alpha_1$-Antitrypsin Deficiency*

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In the classical form of $\alpha_1$-antitrypsin deficiency, a mutant protein accumulates in a polymerized form in the endoplasmic reticulum (ER) of liver cells causing liver damage and carcinogenesis by a gain-of-toxic function mechanism. Recent studies have indicated that the accumulation of mutant $\alpha_1$-antitrypsin Z in the ER specifically activates the autophagic response but not the unfolded protein response and that autophagy plays a critical role in disposal of insoluble $\alpha_1$-antitrypsin Z. In this study, we used genomic analysis of the liver in a novel transgenic mouse model with inducible expression to screen for changes in gene expression that would potentially define how the liver responds to accumulation of this mutant protein. There was no unfolded protein response. Of several distinct gene expression profiles, marked up-regulation of regulator of G signaling (RGS16) was particularly notable. RGS16 did not increase when model systems were exposed to classical inducers of ER stress, including tunicamycin and calcium ionophore, or when a non-polymerogenic $\alpha_1$-antitrypsin mutant accumulated in the ER. RGS16 was up-regulated in livers from patients with $\alpha_1$-antitrypsin deficiency, and the degree of up-regulation correlated with the hepatic levels of insoluble $\alpha_1$-antitrypsin Z protein. Taken together, these results indicate that expression of RGS16 is an excellent marker for the distinct form of “ER stress” that occurs in $\alpha_1$-antitrypsin deficiency, presumably determined by the aggregation-prone properties of the mutant protein that characterizes the deficiency.

The histological hallmark of the classical form of $\alpha_1$-antitrypsin (AT) deficiency is liver cells containing periodic acid-Schiff+/diastase-resistant globules. From many years of research on the disease, we now know that these globules represent rough endoplasmic reticulum (ER) distended by accumulation of the mutant ATZ molecule (where ATZ is the Z variant of $\alpha_1$-antitrypsin). The wild type AT is a classical liver-derived secretory glycoprotein that is delivered by the circulating blood to tissues to subserve its predominant function of inhibiting the neutrophil serine proteases neutrophil elastase, cathepsin G, and proteinase 3. The point mutation that characterizes the ATZ variant converts glutamate 342 to lysine and is sufficient to result in selective retention of the glycoprotein in the ER (reviewed in Refs. 1, 2). Thus, AT deficiency could be considered a prototype, naturally occurring “ER stress” state.

Characterization of the structure of AT and its functional correlates led to the remarkable observation that the substitution of lysine for glutamate 342 conferred on the ATZ molecule a tendency to polymerize and aggregate (3, 4). Although it is still not clear whether the tendency to polymerize is the cause, or an effect, of ER retention, there is clear-cut evidence that polymers and aggregates of this molecule are formed in the ER, and there is growing evidence that these polymers and aggregates play a role in how liver cells respond and whether liver inflammation and carcinogenesis evolve in individuals homozygous for the ATZ allele (2, 5). This is a particularly important issue because we know from unique epidemiological studies carried out in Sweden over the last 35 years that there is wide variability in the liver disease phenotype of individuals that are homozygous for the ATZ allele with only a subpopulation developing severe liver inflammation and/or carcinoma (6, 7). Thus, the Swedish studies implicate genetic modifiers and/or environmental factors that determine the hepatic phenotype of this disease. Cellular response pathways that are activated by the ER stress state are therefore theoretically candidate genetic modifiers or targets of environmental factors that determine the hepatic phenotype. Furthermore,
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cellular response pathways are potential targets for preventive or therapeutic interventions.

In a recent study, we began to characterize the cellular response pathways that are activated by accumulation of ATZ in the ER by using cell line and transgenic mouse models with inducible expression of ATZ (5). We reasoned that these types of model systems would provide highly controlled information about the earliest activation events and would ultimately permit an elucidation of the effect of ER ATZ retention in terms of duration, concentration, and developmental stage. The initial studies in these novel model systems examined known potential response pathways and showed that accumulation of ATZ in the ER activates the autophagic response, the ER overload pathway characterized by activated NFκB, ER, and mitochondrial caspases and BAP31, an ER protein that is thought to mediate pro-apoptotic signals from the ER to the mitochondria. Accumulation of ATZ in the ER did not activate the unfolded protein response (UPR) in the model systems or in human liver tissue (5). We have surmised that this is because of the intrinsic properties of ATZ, perhaps its tendency to polymerize in the ER, because two other naturally occurring nonpolymerogenic AT mutants that accumulate in the ER do activate the UPR. In addition to its importance in understanding the pathogenesis of liver injury in AT deficiency, this last observation provides evidence that ER stress is not synonymous with activation of the UPR, a notion frequently implied in the literature.

The autophagic response appears to be a particularly important part of the cellular response to ATZ accumulation. Our recent studies using cell lines genetically deficient in autophagy show that the autophagic response is essential for disposal of ATZ (8). In the absence of autophagy, inclusions of ATZ were markedly propagated throughout the cytoplasm. Moreover, by using a mouse model with inducible expression of ATZ mated to a mouse model that makes green fluorescent autophagosomes, we found that accumulation of ATZ in the ER is sufficient to activate the autophagic response (8). Further evidence for the importance of the autophagic response has been provided by Kruse et al. (9) who by screening for yeast mutants that were impaired in degradation of ATZ found that autophagy gene atg6 was essential for disposal of the insoluble forms of ATZ. Atg6 was also found to be essential for disposal of mutant fibrinogen subunits that accumulate in the ER in insoluble fibrils in the liver disease associated with inherited hypofibrinogenemia (10). Together, these studies have suggested that the autophagic response is specialized for the type of ER stress that is associated with mutant proteins that are aggregation-prone (11).

In this study, we used genomic analysis of liver from the Z mouse, which has hepatocyte-specific, inducible expression of ATZ, as a method to determine whether there were other cellular response pathways activated by accumulation of ATZ in vivo. Using the inducible system we can titrate the duration, concentration, and developmental stage of the pathologic event, and the results can be interrogated in the context of powerful controls, including Z mice in which the transgene is not induced, M mice with inducible expression of the wild type AT gene in the induced or uninduced condition, and nontransgenic littermates. Because we already know that ER retention of ATZ does not activate the UPR, we hypothesized that the gene expression profiles that were found to characterize ER accumulation of ATZ in this system would identify signaling pathways that have not been previously implicated in ER stress states.

EXPERIMENTAL PROCEDURES

Materials—Rabbit anti-human AT antibody was purchased from Dako (Santa Barbara, CA); goat anti-human AT IgG was from Diasorin (Stillwater, MN); antibody to GAPDH was purchased from U.S. Biochemical Corp.; and antibody to regulator of G protein signaling 16 (RGS16) and GRP78 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Purified human AT was purchased from Athens Research and Technologies (Athens, GA). Tunicamycin and A23187 were from Sigma. Human liver originated from excess liver tissue of normal liver transplant donors and from the native liver of patients with homozygous AT deficiency that underwent liver transplantation as approved by the Human Studies Committee of Washington University School of Medicine, St. Louis.

Transgenic Mice—Mice with hepatocyte-specific inducible expression of wild type AT (M mice), mutant ATZ (Z mice), mutant ATSaar (Saar mice), and mutant ATSaarZ (SaarZ mice) have been described previously (5). These mice were maintained with doxycycline (dox; 40 μg/ml; changed twice weekly) until expression of AT was induced by withdrawal of dox over specified time intervals. The PiZ mouse model with constitutive, tissue-unspecific expression of mutant ATZ (12) bred into the C57/BL6 background has also been described previously (5). For specific experiments tunicamycin was administered intraperitoneally at a dose of 1 μg/g body weight in 150 mM dextrose, and mice were then sacrificed 18 h later for harvesting of the liver, a protocol that has been shown to alter the processing of XBPI1 mRNA in a manner consistent with activation of the UPR (5). Serum samples were obtained by retro-orbital puncture and stored at −20 °C until use. Mice were euthanized by CO2 inhalation. Liver and other tissue were harvested and immediately frozen in liquid nitrogen before storage at −70 °C. All mice were sacrificed in the morning hours unless specified otherwise. Maintenance and all experimental manipulations of the mice were approved by the Animal Research and Care Committee of Children’s Hospital of Pittsburgh, Rangos Research Center.

Cell Lines—Human epidermal HeLa cell lines and murine hepatoma Hepa1–6 cell lines with inducible expression of wild type AT, mutants ATZ, ATSaar, and ATSaarZ have been described previously (5). The HeLa cell lines are named HTO/M, HTO/Z, HTO/Saar, and HTO/SaarZ, and the Hepa cell lines are named HepaTO/M, HepaTO/Z, HepaTO/Saar, and HepaTO/SaarZ. The cell lines were maintained in 40 ng/ml dox until expression of AT was induced by withdrawal of the dox for predetermined time intervals. For experiments with tunicamycin and A23187 HepaTO cell lines were treated for 3 h with tunicamycin (2.5 mg/ml), A23187 (0.25 mM), or solvent control (MeSO4) in an equivalent volume.

Western Blot Analysis and ELISA—For Western blots, serum samples were diluted 1:200 in PBS/Tween 20/Laemmli loading buffer and 10-μl samples loaded on SDS-polyacrylamide gels. Liver samples were homogenized by Dounce homogenizer.
using 3 ml of Argon buffer (50 mm Tris-HCl, pH 8.0, 150 mm NaCl, 0.5% NaDOC, 0.5% Triton X-100). Samples were loaded with 20 μg of total protein for detection of AT and 100 μg for detection of RGS16. Purified human AT (5 ng) was used as a positive control. Blots were blocked in PBS, Tween 20, 5% milk and then incubated with goat anti-human AT or goat antiamouse RGS16 in blocking buffer as first antibody. Horseradish peroxidase anti-goat IgG from The Jackson Laboratory (Bar Harbor, ME) was used as secondary antibody. Blots were visualized with SuperSignal West Dura from Pierce.

For ELISA, mouse serum was diluted 1:10,000 in PBS/Tween 20, 2% bovine serum albumin, 1% milk. Liver homogenates were diluted 1:20 in PBS/Tween 20 (100 mm sodium phosphate, 150 mm NaCl, pH 7.2, 0.05% Tween 20). Total protein concentration was measured using the BCA protein assay kit from Pierce. Purified human AT at dilutions of 1–100 ng/ml was used as standard. 96-Well plates were coated with rabbit anti-human AT, blocked with PBS/Tween 20, and the samples applied. The secondary antibody was goat anti-human AT and then anti-goat IgG horseradish peroxidase conjugate and OPD substrate were used for detection.

High Density Oligonucleotide Array Analysis—Z mice and M mice were studied at ages 6 weeks and 3 months. For each, groups of three mice that had been on or off dox from birth were included. One group of FVB/N mice at the same age was also included as a nontransgenic control. Total RNA was extracted from the liver using TRIzol reagent (Invitrogen) and the RNeasy kit (Qiagen, Valencia, CA). RNA quality control was carried out with the 2100 Bioanalyzer and RNA 6000 NanoLabChip kit (Agilent Technologies). The validity of the experimental paradigm was verified by Western blot analysis from the same livers and Q-PCR from the RNA samples. In each case the levels of AT corresponded to the presence or absence of dox (see below).

cDNA and cRNA synthesis using the liver RNA samples was carried out according to the instructions from Affymetrix. Hybridization to the mouse MOE430A chip set was carried out by the University of Pittsburgh PittArray core facility. Microarray images were analyzed using Affymetrix GCOSS 1.2 software. Signal intensity was scaled to 150 for each microarray. Probe level data (CEL files) were processed by S-PLUS 6.2 array analyzer module (Insightful). Normalization to adjust for background noise, to correct for random and nonspecific binding was done as follows: background control = robust multichip analysis and normalization = quantiles. The differential expression analysis was performed applying local pooled error (LPE) test with Bonferroni adjustment (family-wise error rate/family detection rate control = 0.05).

Expression data of M mice off dox (M4) and M mice on dox (M1) at each age were compared with each other (M4vs1), followed by the same procedure using Z mice off dox (Z4) compared with Z mice on dox (Z1). Intensity ratios were then expressed. Significantly increased and decreased genes ($p < 0.05$) in the wild type (M) group were subtracted from the pool of significantly increased and decreased genes ($p < 0.05$) in the mutant (Z) group, and the fold change threshold was set to 1.8 in juvenile (6-week-old) and 2.0 in adult (3-month-old) comparisons (Fig. 2, analysis strategy). Hierarchical clustering was carried out with the Genes at Work software (13). Pathway analysis was carried out by subjecting the data set containing differentially expressed genes to analysis for relevant molecular interactions, biological functions, and pathways using the Ingenuity Pathway Analysis tool. Comparison between the two groups was carried out with unpaired t test and/or Mann-Whitney Rank sum test (SigmaStat 2.03).

Real Time Quantitative PCR—Total RNA was purified from mouse liver using RNeasy columns (Qiagen); three livers per condition were harvested. First strand cDNA (RT reaction) was synthesized from 500 ng of RNA using Superscript II (Invitrogen) according to the manufacturer’s directions. A negative control was also performed without enzyme (NRT reaction). RT and NRT reactions were also performed on 500 ng of commercially prepared liver RNA (Ambion, Austin, TX) to serve as the calibrator for the real time QPCRs. Each experimental sample was normalized to a nontransgenic control (fold change).

For PCR, duplicate aliquots of the RT reaction and 1 aliquot of the NRT reaction served as templates for the target genes and the control gene $\beta$-glucuronidase. Reaction components, including the ROC internal control, were obtained from Applied Biosystems (Foster City, CA). The probes and primers were obtained from Applied Biosystems (FAM$^\text{TM}$ dye labeled TaqMan$^\text{TM}$ minor groove binder probes, inventoried assays). Real time reactions were run on an ABI7000 and ABI7300 with the following cycle conditions: 95 °C for 12 min (95 °C for 15 s and 60 °C for 1 min) for 40 cycles. Differential gene expression was calculated by the $\Delta \Delta C_T$ calculation (14). The $\Delta C_T$, method controls for potential differences in efficiency of the RT, as well as the PCR, whereas calculations based on standard curves do not ($\Delta \Delta C_T = \Delta C_T(\text{expr}) - \Delta C_T(\text{nexp})$ when $\Delta C_T = C_T(\text{gene}) - C_T(\text{norm})$).

RESULTS

Gene Expression Profile in the Liver in Response to Accumulation of ATZ in Hepatocytes—To ensure that the model system was appropriate, liver was harvested from 3-month-old Z mice and mice that had been on or off dox since birth, and then subjected to Western blot analysis for AT (Fig. 1). The results show that the AT polypeptide is detected in equivalent amounts in the Z and M mice but only when off dox. There was no detectable AT when either mouse had been on dox. Control included equivalent amounts of liver from a normal human specimen, a specimen from a patient homoyzgous for AT deficiency, and the PiZ mouse that has ubiquitous constitutive expression of human ATZ (5). These controls show that the AT expressed in the Z and M mice migrates to the same electrophoretic mobility and is present at the same or lower steady state levels indicating that
it is not expressed at supra-physiologic levels in these models. Absence of a signal in liver from the FVB/N mouse, the background strain for the Z and M mice, provides reassurance that the Western blot analysis is done under conditions that do not detect the endogenous murine AT ortholog.

Next, high density oligonucleotide array analysis of hepatic genes was carried out in Z mice at 6 weeks of age (“juvenile”) and 3 months of age (“adult”). Groups of three mice each had (Z1) or did not have dox (Z4) in the drinking water. Controls included M mice on (M1) or off (M4) dox and nontransgenic mice from the background strain FVB/N. RNA and protein were extracted from the liver. Changes in AT expression were first validated by Western blot analysis, ELISA, and real time QPCR. RNA was then hybridized to the mouse Affymetrix MOE430A chip set, and the resulting data were subjected to differential expression analysis with the LPE test (S-Plus).

Changes in gene expression in the Z4 group versus the Z1 group were compiled. Several criteria were used to conclude that these changes were significant. First, they needed to be greater than 1.8-fold in magnitude. Second, the change needed to be statistically significant. Third, there could not be a similar change in gene expression in the M4 versus the M1 group. Fourth, any significant change in gene expression in the Z4 group compared with the Z1 group needed to be significantly changed in the same way (up-regulated or down-regulated) in the Z4 compared with the M4 and the Z4 compared with the nontransgenic FVB/N group (Fig. 2).

Using these criteria, 25 up-regulated transcripts and 24 down-regulated transcripts were identified in the juvenile series and 75 up-regulated transcripts and 131 down-regulated transcripts were identified in the adult series (supplemental Table 1). Six transcripts were up-regulated, and seven transcripts were down-regulated in both juvenile and adult series.

There was marked up-regulation of human AT gene expression in Z4 versus Z1 and in M4 versus M1 reaching between 3040- to 8855-fold, providing validation of the induction of AT gene expression. Several patterns of global gene expression changes were notable. The gene expression profile did not reflect an unfolded protein response in any substantive way. The only change in gene expression that was similar to what is seen in the UPR was a 2.4-fold increase in HERPUD1 in the adult series. No other changes in expression of genes known to be altered during the UPR or that appear in previous high density oligonucleotide array analyses of the UPR (15–20) reached significance in this study, including absence of the 2.5–3-fold increase in GRP78 and GRP94 and 3.1–3.9-fold increase in protein-disulfide isomerase and its related proteins.

The profile of changes in gene expression was analyzed in three ways. First, using the Affymetrix GO Browser software, there were significant changes in 19 categories, the most significant of which were regulation of transcription, lipid/steroid/sterol biosynthesis and metabolism, other biosynthesis/metabolism, cell cycle/proliferation and differentiation/cell fate, signal transduction/signaling, and immune response (supplemental Table 2). Other categories that had significant changes were regulation of protein synthesis, protein and amino acid phosphorylation events, proteolysis, ubiquitin-dependent processes, protein folding, carbohydrate metabolism, electron transport, other transport, circadian rhythm, regulation of apoptosis, cell adhesion, and cytoskeleton.

Second, using the statistical analysis program EASE, a large number of biological themes reached significance, most of which overlapped with the GO analysis. Using clustering analysis, the EASE program identified three major clusters in the up-regulated genes and five major clusters in the down-regulated genes. The clusters of up-regulated genes were in categories of regulation of transcription/cellular metabolism/biological rhythm/ubiquitin conjugation (enrichment = 2.18); heme-binding/iron-binding/oxidoreductase/linoelic acid metabolism (enrichment = 1.56); protein-tyrosine kinase/phosphotransferase/ATP-binding/protein serine-threonine kinase (enrichment = 0.66). The clusters of down-regulated genes were in categories of cholesterol and lipid metabolism (enrichment = 7.44); protein metabolism/ubiquitin conjugation/lipoprotein metabo-
FIGURE 3. Biological themes deduced from genes significantly altered in expression in response to accumulation of mutant ATZ. A, molecular and cellular functions; B, metabolic and signaling pathways. The level of significance shown on the vertical axis was determined by the p value of the right-tailed Fisher’s exact test using Ingenuity Network Analysis software.
The two networks with the highest number of significant gene expression changes are shown. The networks are displayed graphically as nodes (genes/gene transcription/cell metabolism/zinc-binding/TGFβ signaling (enrichment = 1.65)).

Third, we used Ingenuity Pathway Analysis software. Significant changes in gene expression were found in a number of categories of molecular and cellular function (Fig. 3A) that were also identified by the GO and EASE programs. Four metabolic and signaling pathway categories were identified, including sterol biosynthesis, glycine-serine/threonine synthesis, antigen presentation, and cytochrome P450 pathways (Fig. 3B).

We also used Ingenuity Pathway Analysis to predict networks that are affected by the gene expression profile. This analysis identified 11 networks. The two most powerful of these suggest a marked effect on cellular growth and proliferation/carcinogenesis converging on the SP1, hepatic nuclear factor 4α, Kruppel-like factor 10 transcription factors, bone morphogenetic factor 2 (Fig. 4A), and a network showing a marked effect on cellular morphology and cellular compromise converging on tumor necrosis factor superfamily member 2 and fibroblast growth factor 19 (FGF19) (Fig. 4B). Interestingly, the effects on tumor necrosis factor superfamily member 2 and FGF19 were not apparent in the gene expression profile and therefore predict post-transcriptional changes in activity of these proteins. The other networks predicted by the Ingenuity Pathway Analysis include the following (supplemental Fig. 1): changes in lipid metabolism that would be explained by a decrease in sterol regulatory element-binding transcription factors 1 and 2 (SREBP1 and SREBP2) activity (supplemental Fig. 1a); changes in cellular movement/morphology/assembly that predict an increase in mitogen-activated kinase (ERK2) and ERBB2 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 2) activity (supplemental Fig. 1b); changes in cell death/carcinogenesis that predict increases in caspase 3 and apoptotic peptidase activating factor 1 activity together with increased transcription factor CEBPβ and cyclin-dependent kinase inhibitor 2a activity (supplemental Fig. 1c); a network that converges on increased p53, TNFα-induced protein 2 and TGFβ (transforming growth factor β) activity (data not shown); a network that predicts increased myc and interferon-γ activity (data not shown); and a network that predicts increased NFκB activity with increases in several inflammatory cytokines and chemokines (data not shown).

Selected genes that were significantly changed in expression are shown in supplemental Tables 3 and 4. These genes were selected because they were of obvious potential biological significance and/or were changed at both time points and/or were confirmed by real time QPCR. Among the up-regulated transcripts (supplemental Table 3) are several important groups, including transcription factors (D-site albumin promoter-binding protein, period homolog 3, TAR(HIV) RNA-binding protein, Kruppel-like factor 10, activating transcription factor 2, nuclear receptor interacting protein 1, thymotroph embryonic factor, SP1, CCNRN, zinc finger protein 106, hepatic nuclear factor 4α, BCL6), important signaling molecules (interferon-activated gene 203, interleukin 6 receptor α, RGS16, RGS5, serum deprivation response), growth factors (connective tissue growth factor, leukocyte cell chemotaxin 1), components of the ubiquitin system (ubiquitin specific protease 2 and 14, homocysteine-inducible, endoplasmic reticulum stress inducible, ubiquitin-like domain member 1, HERPUD1), kinases and phosphatases (proviral integration site 3, lipin 2, protein tyrosine phosphatase 4a2, p85α), and ER proteins (cytchrome P450s, cyclophilin F, polycystic kidney disease 2). Among the down-regulated transcripts (supplemental Table 4) are several transcription factors (aryl hydrocarbon receptor nuclear translocator-like, inhibitor of DNA-binding 3 (ID3), nuclear factor/interleukin-3-regulated, early growth response 1, activating transcription factor 5, CCR4-NOT transcription complex subunit 7, signal transducer and activator of transcription 1, STAT1), signaling molecules (arginine vasopressin receptor 1A, monocyte to macrophage differentiation-associated 2, tumor necrosis factor α-in-
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duced protein 2, T-cell specific GTPase, CD36), ER proteins (presenilin 2, UDP glucuronosyltransferase, cytochrome P450s, sterol C5 desaturase homolog, 3-hydroxymethyl-3-methylglutaryl-coenzyme A synthase, sterol C4 methyl oxide-like, TAP-binding protein, peptidylprolyl isomerase B), and lysosomal enzymes (cathepsin Z, cathepsin C, mannosidase 2αB2).

Several patterns are noteworthy. First, a number of the transcription factors that are up-regulated are known to be involved in liver-specific gene expression and in transcriptional regulation of circadian gene expression (D-site of albumin promoter-binding protein, period homolog 3, thyrotroph embryonic factor, nuclear factor/interleukin 3-regulated, CCR4-NOT). Second, a number of the changes in gene expression are indicative of a TGF-β effect (SP1, activating transcription factor 2, connective tissue growth factor, early growth response 1, leukocyte cell-derived chemotaxin 1, Kruppel-like factor 10, ID3). This would be consistent with the stereotypic fibrotic response of liver to injury. Furthermore, the decrease in procollagen type IIIα1 is consistent with the fibrous tissue composition changes that accompany severe liver disease. Third, many genes that could alter lipid metabolism were significantly altered in expression, consistent with the mild-to-moderate steatosis that is observed in the liver in AT deficiency. Fourth, the changes in components of the ubiquitin and proteasomal system are interesting for increases in deubiquitinating enzymes and a decrease in a proteasomal regulatory subunit. Fifth, changes in gene expression that would be predicted to affect cell proliferation and cell death are particularly abundant.

**Up-regulation of RGS16 Gene Expression in the Liver in Response to ER Accumulation of ATZ**—We were particularly interested in the changes in expression of RGS16 that were observed in the genomic analysis for several reasons. First, it was significantly increased in both juvenile and adult mice. Second, the magnitude of its increase in the adult mice was among the most significant. Third, it was accompanied by an increase in another RGS family member, RGS5, even though the increase in RGS5 was of lesser magnitude. Fourth, up-regulation of RGS16 is potentially of great functional significance. It is known to bind to and inactivate Gα13 (21), and a recent study in a Gα13 knock-out mouse model provides evidence that Gα13 mediates the hepatic anti-autophagic effect of TOR kinase (22). Thus, up-regulation of RGS16 may be one of the ways that accumulation of ATZ in the liver activates the autophagic response.

First, we used real time QPCR to validate the results of the genomic analysis with respect to RGS16 (Table 1). The results indicated that there was a marked and specific induction of RGS16 mRNA levels.

Second, we used Western blot analysis to determine whether RGS16 protein levels were increased in the liver when ATZ expression was induced (Fig. 5). The results showed a significant increase in steady state levels of RGS16 in the liver of Z mice after 3 months off dox compared with the liver of Z mice that were still on dox. The increase was seen at several times during the day, excluding the possibility that it was a result of diurnal variation or differences in feeding. The increase was specific as shown by the lack of change in the control GAPDH.

**RGS16 Up-regulation Is Specific for the ER Stress State Associated with ATZ Accumulation**—To determine whether the effect on RGS16 was specific we used real time Q-PCR for RGS16 mRNA levels after administration of tunicamycin, a classical ER stressor, in vitro. Nontransgenic FVB/N mice, M, Saar, and Z mice with AT expression suppressed by dox administration were subjected to 12% SDS-PAGE and then Western blot analysis for RGS16 (top) or for GAPDH (bottom).

**TABLE 1**

| Group                  | FC + S.D. | p (Student’s t test) |
|------------------------|-----------|----------------------|
| Z no DOX juv           | 5.5 ± 0.46| <0.001               |
| Z DOX juv              | 0.28 ± 0.08|                      |
| Z no DOX adult         | 19.8 ± 7.4| 0.011                |
| Z DOX adult            | 0.44 ± 0.63|                      |

**TABLE 2**

| Group                  | FC + S.D. |
|------------------------|-----------|
| FVB/N                  | 0.19 ± 0.10|
| M no DOX adult         | 0.06 ± 0.05|
| Z DOX adult            | 0.19 ± 0.20|
| Saar no DOX adult      | 0.03 ± 0.02|
| Saar no DOX juvenile    | 0.03 ± 0.01|

* FC, indicate fold change compared with background strain (FVB/N).
DISCUSSION

In this study a mouse strain with hepatocyte-specific inducible expression was used as a model for genomic analysis of the hepatic response to accumulation of mutant ATZ in the ER, the pathologic state that characterizes the classical form of AT deficiency. The results provide further evidence that this pathologic state elicits a distinct form of ER stress and, perhaps even more importantly, that distinct signaling pathways and changes in gene expression may be activated depending on distinct characteristics of the ER stress state. One of the most important distinctions is the absence of changes in gene expression that would be expected of an unfolded protein response. The only change in gene expression that overlaps with what is expected of an unfolded protein response was a 2.4-fold increase in HERPUD1 in adult mice only. There was no increase in GRP78, GRP96, CHOP, or activating transcription factor 6 target genes. The lack of an unfolded protein response is entirely consistent with our previous studies in cell line models and in the liver of mouse models and human patients targeting specific markers of the UPR (5) as well as studies done in other laboratories using a variety of systems (24, 25). It is not yet known why the UPR is not activated when ATZ accumulates in the ER, but it appears to be attributable to the polymerogenic properties of ATZ because the UPR is activated when the nonpolymerogenic truncated variants of AT (ATSaar and ATSaarZ) accumulate in the ER (5).

A recent study by Bernales et al. (26) shows that autophagy is activated in yeast as a result of the UPR, using administration of dithiothreitol or tunicamycin. Data from that study provided evidence for the concept that sequestration of parts of the ER by autophagosomes somehow offsets the expansion of the ER that accompanies the UPR, presumably reaching a steady state level at a larger ER size. Using this paradigm one could imagine a sequence of events in AT deficiency, and in systems modeling AT deficiency, with an initial response to the accumulation of ATZ in the ER that involves the UPR followed by activation of autophagy and then a steady state of ATZ accumulation and sequestration in autophagosomes is reached. Furthermore, once this steady state is reached there might be no further evidence for the changes in gene expression that characterize the UPR. Although we cannot completely exclude it, several observations militate against this possible sequence of events. First,
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![Figure 7. RGS16 gene expression in liver from patients with AT deficiency. RNA and protein were extracted from liver of controls (5) and patients with homozygous ZZ AT deficiency (8). The controls included four normal liver donors and one patient with biliary atresia. The RNA was analyzed for RGS16 mRNA levels by QPCR, and protein was analyzed by Western blot analysis for AT levels with or without being separated into soluble and insoluble fractions. Total and insoluble AT levels are shown.](image)

there was no evidence for UPR-type gene expression changes in the juvenile mice here. Second, we did not detect any changes in gene expression compatible with the UPR very shortly after induction of ATZ gene expression in cell line models in our previous studies (5). In either case, if up-regulation of RGS16 is an important mediator of the autophagic response associated with ATZ, as suggested here, then its appearance after accumulation of ATZ but not after administration of tunicamycin and A23187 appears to distinguish the two situations.

One of the most interesting signatures of the response to the ER stress engendered by ATZ is marked up-regulation of RGS16. Up-regulation of RGS16 in the liver was observed at age 6 weeks and increased by 3 months of age. It was apparent at the protein level as well as at the mRNA level. This is particularly important because steady state levels of RGS16 protein are regulated by ubiquitin-dependent proteolysis that involves the N-end rule pathway (27). Elevation in its expression could not be attributed to diurnal variation or timing of feeding. RGS16 was induced in cell line models and in the liver of mouse models when expression of ATZ was induced but not when expression of the nonpolymerogenic mutant AT Saar was induced, indicating that it was specific for ER accumulation of ATZ. Furthermore, RGS16 was not induced by conditions known to induce the UPR, treatment of cell lines with tunicamycin or A23187, or treatment of mice in vivo with tunicamycin. Finally, RGS16 mRNA levels were markedly increased in the liver of patients with AT deficiency, and variation in those levels among the deficient individuals was correlated with steady state levels of insoluble ATZ protein. These data suggest that induction of RGS16 gene expression is specific for the ER stress state that is associated with the ATZ protein and perhaps even more specifically for the accumulation of aggregated protein in the ER.

Several observations raise the possibility that induction of RGS16 might be one mechanism by which accumulation of ATZ in the ER activates the autophagic response. First and foremost, a recent study by Gohla et al. (22) has shown that targeted disruption of the heterotrimeric G protein Gαi3 in a mouse model leads to a marked increase in hepatic insulin-induced autophagic activity. This means that Gαi3 mediates the hepatic anti-autophagic effect of insulin. Because RGS16 binds to Gαi3 (21) and is thought to inhibit G signaling, one could hypothesize that induction of RGS16 when ATZ accumulates in the ER serves to inhibit a Gαi3-mediated signaling pathway and, in so doing, de-represses autophagy. Further evidence for this idea comes from studies of another member of the RGS family, AGS3. AGS3 has a GoLoco domain in its carboxyl terminus that shares extensive homology with a domain in RGS16. This domain of AGS3 binds Gαi3, prevents the anti-autophagic action of Gαi3, and stimulates autophagy (28).

Fasting leads to marked induction of hepatic RGS16 gene expression (29) and is a known stimulus of autophagic activity. Thus we could envision RGS16 as a potential mediator of the autophagic response that is activated by ER accumulation of ATZ and that is designed for disposal of aggregated proteins. In contrast, when a soluble unfolded protein like AT Saar accumulates in the ER, RGS16 is not induced. Rather the UPR and the ubiquitin-dependent proteasomal system is activated and presumably plays a key role in disposal of soluble unfolded mutant protein. It is important to point out the complexities that will need to be addressed in addressing the potential functional consequences of RGS16 up-regulation. It is possible that up-regulation of RGS16 is only one of several mechanisms by which autophagy is activated in AT deficiency and that it is not sufficient for activation of autophagy. Indeed, we already know that RGS5 expression is up-regulated and that RGS5 has some Gαi3 antagonistic activity (21).

A number of the other changes in gene expression are consistent with what is known about the effects of AT deficiency in the liver. First, a number of changes in gene expression are consistent with marked effects on regulation of cell proliferation/cell death and carcinogenesis. For example, there is up-regulation of activating transcription factor 2, SP1, CCR4-NOT transcription complex subunit 7, PKD2, COP9, spindlin, TIMP2, and proviral integration site 3 together with down-regulation of early growth response 1,
STAT1, activating transcription factor 5, p85α, dynen light chain Tcxt-type 1, IL3-regulated nuclear factor, suppressor of tumorigenicity 5, and ID3. Furthermore, network analysis suggests that ERK2, caspase 3, apoptotic peptidase activating factor 1, cyclin-dependent kinase inhibitor 2a, p53, Fos, and Myc functional activity is activated. Activation of ERK2 has recently been shown to play a key role in hepatocyte replication after partial hepatectomy (30). Effects on expression of genes that alter cell proliferation and carcinogenesis are completely consistent with our recent observations that accumulation of ATZ in hepatocytes has a profound effect on cell proliferation and cell death and engenders a cancer-prone proliferative state on hepatocytes with lesser accumulation of ATZ in the liver in AT deficiency (31, 32). Second, a number of changes in gene expression are indicative of a TGFβ effect, including increased SP1, activating transcription factor 2, connective tissue growth factor, leukocyte cell chemotaxin 1, Kruppel-like factor 10, and decreased early growth response 1, ID3 together with decreased procollagen type IIIα1. These changes are entirely consistent with the known hepatic fibrotic effect of AT deficiency. Third, there were numerous changes in gene expression that were indicative of a marked effect on sterol and lipid metabolism. The Ingenuity Network Analysis predicts that many of these changes can be explained by decreases in SREBF1 and SREBF2. Although it is not as marked as seen in nonalcoholic steatohepatitis, hepatic steatosis is certainly observed in AT deficiency, and mitochondrial dysfunction is believed to be one of the final common pathways for cell compromise in this disorder (33). Changes in sterol and lipid metabolism are also a critical part of the classical mechanisms by which liver attempts to regenerate (34), a process that is believed to be influencing the globule-devoid hepatocytes in AT deficiency. Fourth, network analysis predicts increased NFκB activity and associated increases in the activity of a number of inflammatory cytokines and chemokines, including IL-4, IL-9, CCL5, CCL6, CXCL12, CCL7, and CCL22 together with interferon activated gene 203 and toll-like receptor 2 activity. Our previous studies have shown marked increases in NFκB DNA binding activity in cell lines that accumulated ATZ as well as in the liver of mouse models of AT deficiency. Increased NFκB activity could explain the known neutrophilic infiltration that occurs in the liver in AT deficiency and could participate in the pathobiology of hepatocellular carcinogenesis. Increased IL-4, IL-9, and CCL5 activity would be consistent with a Th2 lymphocytic type inflammatory process.

However, several other patterns of gene expression have not been previously considered in the hepatic pathobiology of AT deficiency. First, there are marked changes in the expression of cytochrome P450 isozymes as well as a number of other ER proteins. This type of effect could reflect changes in ER composition needed for membrane proliferation and expansion to accommodate the accumulating mutant protein load. Second, the changes that are likely to impact the ubiquitin-dependent proteasomal pathway included increases in expression of two de-ubiquitinating enzymes and a decrease in one of the proteasomal regulatory subunits. Third, there were significant decreases in expression of a surprising number of lysosomal enzymes. Fourth, changes in expression of a cluster of transcription factors that play a role in liver-specific gene expression and are regulated by circadian rhythm were striking. Together with the up-regulation of RGS16 and activation of the autophagic response, these results raise the possibility that accumulation of an aggregated protein gives the cell the impression that it is being starved and results in a cellular response program that has many of the features of the response to starvation. Fifth, the gene expression profile suggests the involvement of a number of other signaling pathways that could play a role in AT deficiency-associated liver disease, including genes that are involved in the insulin-like growth factor 1 signaling pathway, mitogen-activated protein kinase, and stress-activated protein kinase pathways as well as the hypoxic and xenobiotic response pathways. The implication of these pathways should lead to several new hypotheses for explaining the etiopathogenesis of hepatic injury and/or carcinogenesis in AT deficiency.

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