Regulated de novo biosynthesis of fibrinogen in extrahepatic epithelial cells in response to inflammation

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Summary

Hepatic fibrinogen (FBG) is upregulated during an acute phase response (APR) induced by glucocorticoids and interleukin (IL)-6. Furthermore, intestine and lung epithelium synthesize FBG after exposure to inflammatory mediators, and both plasma and lung cell-derived FBG, along with fibronectin, assemble in detergent-insoluble extracellular matrices (ECM) of pneumocytes and fibroblasts independent of thrombin or plasmin cleavage. An epitope cryptic in soluble FBG (β15-21) but exposed in matrix-FBG and fibrin induces cell proliferation and actin cytoskeleton reorganization during wound repair and angiogenesis. Although fibrinogen is involved in hemostasis and homeostasis, mechanisms regulating extrahepatic FBG expression remain unexplored. Herein we examined FBG production by lung compared to liver epithelial cell lines in response to dexamethasone (DEX)+IL-6. Regulated synthesis of HepG2-FBG follows the pathway shown for constitutive synthesis by liver epithelium. Constitutive A549-FBG expression was not detectable, however, intracellular FBG precursors in DEX+IL-6-treated A549 lung cells were similar to HepG2 cells with two notable exceptions. The relative rate of chain synthesis in HepG2 cells was unequal, whereas nascent synthesis of all three chains occurred at equivalent rates in stimulated A549 cells. Unlike HepG2 cells, which rapidly secreted intact FBG, nascent dimeric FBG accumulated in the A549 cell-associated fraction prior to release into medium. Furthermore, soluble A549-FBG was susceptible to thrombin and plasmin cleavage. Interestingly, many functionally diverse proteins possess FBG-related domains that direct cell-fate determination during development or wound repair, suggesting that extrahepatic FBG biosynthesis evoked only during inflammation plays such a role during localized injury and repair to restore tissue homeostasis.

Keywords
Extrahepatic fibrinogen, wound repair, lung epithelium, thrombin, extracellular matrix

Introduction

The predominant form of vertebrate fibrinogen (FBG) is a complex dimeric protein of approximately 340,000 M₉, that is composed of pairs of 3 nonidentical polypeptide chains designated Aα, Bβ and γ. These polypeptide chains are assembled by intra- and inter-chain disulfide-bonds to form a complex trinodular structure linked by two coiled-coil regions (reviewed in (1, 2)). FBG is a principal factor in the maintenance of hemostasis, and during disruption of homeostasis, FBG is upregulated as part of the systemic acute phase response (APR) (3). The liver is the primary source of plasma FBG, however, several studies demonstrate expression and synthesis of FBG in epithelial cells of extrahepatic tissues (4-11). The production of FBG by lung (8, 9) and intestinal (11) epithelium requires an inflammatory stimulus, which can be
provided experimentally by IL-6 and dexamethasone (DEX). Unlike hepatocytes that constitutively produce significant amounts of FBG, there is little detectable constitutive expression of FBG in lung epithelial cells in the absence of DEX+IL-6 treatment (8, 12, 13). Taken together, these results suggest that the ubiquitous expression of the γFBG gene in extrahepatic tissues is not coordinated with expression of the FBG Aα and Bβ chain genes under basal conditions resulting in a cell-type specific difference in nascent FBG production.

Although both lung-A549 and liver-HepG2 lines are derived from epithelial cells, morphometric and electron microscopic studies indicate organ-specific functional differences. A notable difference between lung and liver epithelium is the degree to which these cell types exhibit polarity from the apical to the basolateral face of the cell. Liver architecture is characterized by continually curving plates of hepatocytes bounded by sinusoids into which nascent plasma proteins are secreted to enter systemic circulation. In contrast, lung epithelium is highly polarized separating the airway from the abluminal basement membrane and capillary bed. After DEX+IL-6 induction, nascent FBG is secreted from the basolateral face of A549 cells (13) and assembled into the extracellular matrix (ECM) (12), reflecting the distinct barrier function of lung architecture. In HepG2 cells however, FBG is secreted in equivalent amounts from both apical and basolateral surfaces (13), consistent with rapid secretion into circulation.

The functional importance of plasma FBG and fibrin in both normal and pathophysiologic mechanisms of disease has been extensively studied. However, there is a paucity of information regarding both the structure and function of FBG produced by extrahepatic epithelium. In this report, we examined the pattern of biosynthesis and assembly of FBG in response to DEX+IL-6 and the structure-function similarities or differences between FBG produced by hepatocytes and pneumocytes, focusing our studies on FBG produced by lung alveolar epithelial cells as a model for extrahepatic synthesis of FBG. Taken together with our earlier reports (8, 9, 12-15), the results of this study support the hypothesis that the regulated synthesis, polarized secretion and deposition of FBG into the ECM alveolar epithelium during inflammation contribute to the maintenance of alveolar epithelial homeostasis.

Materials and methods

Cells, culture conditions, metabolic labeling and immunoprecipitation

Human HepG2 liver and A549 lung cell lines were obtained from the American Tissue Culture Collection (Rockville, MD, USA) and grown as previously described (8). For induction of FBG synthesis, cells were stimulated with 2.5 ng/ml IL-6 and 0.1 μM DEX (8). Recombinant human IL-6 was purchased from Gibco-BRL (Grand Island, NY, USA) and DEX was from Sigma (St. Louis, MO, USA). In some experiments, HepG2 and A549 cells were continuously labeled with 40 μCi/ml [35S]Met+[35S]Cys EasyTag Express Protein Labeling Mix (DuPont NEN, Boston, MA, USA) in the absence or presence of DEX+IL-6 in complete culture medium for 18 hr. In other experiments, pulse-chase labeling of nascent proteins was carried out after an 18-hour induction with DEX+IL-6. Conditioned media were removed and cell monolayers were washed and starved for 15 min in serum-free medium. The cells were pulsed for 3 min with 1 μCi/ml EasyTag in serum-free medium (16). After removal of the pulse-labeling medium, the labeled proteins were chased with isotope-free complete medium for varying intervals of time. Conditioned media were collected, supplemented with 100 U/ml aprotinin (CalBiochem, LaJolla, CA, USA) and immunopurification of FBG carried out as previously described (8, 17, 18).

Electrophoretic analysis of immunopurified FBG

After immunopurification, samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 8% gels according to Laemmli as described in detail (19). For 2D-gel electrophoresis, nonreduced protein was resolved in the first dimension on horizontal 2% agarose slab gels prepared in 0.1% SDS and 50 mM phosphate buffer, pH 7.0; the gel running buffer consisted of 100 mM phosphate buffer, pH 7.0 with 0.1% SDS. After resolution in the first dimension, each sample lane was cut-out and incubated with constant shaking for 20 min at room temperature in Laemmli running buffer with 50 mM dithiothreitol to reduce the disulfide bonds between the individual chains of the FBG molecule (20). The agarose strip was transferred to the top of an 8% polyacrylamide gel and overlaid with 0.5% agarose with 0.1 mM dithiothreitol for the second dimension electrophoresis in the discontinuous buffer system of Laemmli. Continuous buffer Weber-Osborn SDS-PAGE was carried out using 7% gels as recently described in detail (19). Electrophoresis reagents were purchased from BioRad (Hercules, CA, USA). Fluorography was carried out on gels equilibrated in EnHance (DuPont NEN) prior to drying. Scanning densitometry was performed using NIH Image 1.62 software and the intensity of each band was normalized to the total number of Met and Cys in each band.

Biochemical analysis of lung epithelial cell FBG

To examine N-linked glycosylation of FBG, tunicamycin was added during the last 4 hours of incubation with DEX+IL-6. The cells were pulse-labeled for 3 min with EasyTag labeling mix at 1 μCi/ml in serum-free medium followed by a 90 min chase in complete medium with 10 μg/ml tunicamycin (CalBiochem) ± DEX+IL-6. Changes in the glycosylation pattern of the FBG Bβ and γ chains were visualized by reducing
SDS-PAGE and fluorography. To determine whether lung epithelial cell-derived FBG was susceptible to thrombin or plasmin cleavage, A549 cells were metabolically labeled with 40 µCi/ml EasyTag in DEX+IL-6 for 18 hours. Following immunoprecipitation from conditioned media, FBG bound-Sepharose beads were washed and resuspended in 0.1M Tris-HCl, pH 8.3 plus 50 mM CaCl₂ and cleaved with 2 U/ml thrombin (CalBiochem) for 1 hr at 37°C. The digests were stopped with 1 U/ml hirudin (Sigma) and were analyzed under reducing conditions by Weber-Osborn SDS-PAGE on 7% gels containing 0.8 M urea for fluorography. For Western blotting, samples were resolved on Weber-Osborn gels without the urea (19). The membranes were probed with monoclonal antibody T2G1 (Accurate Chemicals), which is specific for the fibrinogeneitope β₁₅-₂₁ exposed upon thrombin cleavage of the Bβ chain of FBG (21), at a concentration of 10 µg/ml followed by horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:1500) (Molecular Probes, Eugene, OR, USA); immunoreactive bands were detected with chemiluminescence reagents (DuPont NEN). Plasmin (CalBiochem) was added (1.0 CTA U/ml) to immunopurified metabolically labeled FBG in 0.1 M Tris-HCl pH 8.3 and incubated at 37°C for 6 hr. Aprotinin was added to a final concentration of 100 U/ml to stop plasmin activity. FBG and plasmin generated FBG fragments were resolved by SDS-PAGE on 5 to 9% gradient gels under non-reducing conditions in Laemmli buffer.

**Statistical analysis**

The experiments were repeated three to six times and significance (p-value <0.05) determined by two-way ANOVA (StatView™ Abacus Concepts Inc., Beverly, CA, USA).

**Results**

**Composition of nascent secreted FBG**

Previous studies have shown that nonstimulated HepG2 cells contain intracellular pools of free Aα and γ chains of FBG (16, 22-24). In response to acute or chronic inflammation, the synthesis of plasma FBG is upregulated 2 to 10-fold; however, it is not known whether the pattern of human FBG assembly in

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**Figure 1:** Effects of DEX±IL-6 treatment on the composition of nascent FBG produced by HepG2 and A549 cells. Panel A. HepG2 and A549 cells were treated with DEX+IL-6 for 18 hr, starved for 15 min, pulse-labeled with [³⁵S]Met+[³⁵S]Cys EasyTag mixture then chased for 30 min intervals up to 120 min with label-free complete medium. Control HepG2 cells were processed similarly in the absence of DEX+IL-6 treatment. The nonstimulated A549 control condition was not performed due to the low constitutive level of FBG production observed previously (8, 9, 12, 13). A representative fluorograph (n=4) of reduced and denatured immunopurified FBG resolved on an 8% polyacrylamide gel is shown. The numbers above the figure correspond to the chase intervals. Panel B. Control HepG2 (left panel) and DEX+IL-6-treated HepG2 (middle panel) and A549 (right panel) cells were metabolically labeled during the 18 hr treatment after which radiolabeled media were removed and cell monolayers were washed to remove unincorporated label. Fresh complete medium was added to cells and the amount of FBG secreted in 1 hr was measured by immunoprecipitation, SDS-PAGE and densitometric scanning of resulting fluorographs (n=4); densitometric analysis was normalized for relative content of Met + Cys in each chain. This step was repeated at 1 hr intervals for 7 hr as indicated above each panel. The positions of migration of the Aα, Bβ and γ chain isoforms is denoted.
hepatocytes is the same during the inflammatory response. To determine whether intracellular pools of Aα and γ chain polypeptides are formed in response to an inflammatory stimulus, A549 and HepG2 cells were treated with DEX+IL-6 for 18 hours, pulse-labeled, then chased for 30 min intervals up to 120 min with label-free complete medium and nascent secreted FBG was immunopurified for SDS-PAGE and fluorography (Fig. 1A). The radioactivity of each polypeptide chain was normalized to account for the relative number of Met and Cys present in each chain (Table 1). If free pools of intracellular Aα and γ chains are found after 18 hr stimulation with DEX+IL-6, we would expect to find only the Bβ polypeptide chain significantly radiolabeled with faint labeling of the Aα and γ chains in the intact FBG molecules secreted during the first 30 min chase period. At later time points once the preformed (nonlabeled) pools of Aα and γ chains were exhausted, newly synthesized (labeled) Aα and γ chains would be assembled with nascent (labeled) Bβ chain into intact FBG and secreted. On the other hand, if there is no intracellular pool of Aα or γ chains, then all three polypeptide chains will be metabolically labeled at saturating density, i.e., equivalent relative specific activities. The results indicate that after 18 hours of DEX+IL-6 treatment, intracellular pools of free (faint- or non-labeled) Aα and γ chains exist in both A549 and HepG2 cells (Fig. 1A, Table 1); the labeling density of the Aα and γ chains increases with time of chase such that there is no significant difference in the relative specific activity of the Aα, Bβ and γ chains in FBG secreted by 90 min (Table 1). A higher labeling density in the Bβ chain was consistently found at the earlier time points regardless of the cell type, treatment condition and chase intervals, compared to a lower labeling density in the γ chain bands, suggesting that neither cell-type nor treatment changes the order of FBG chain synthesis and assembly into FBG for secretion.

Another set of experiments was performed to examine the loss of each FBG chain after continuous metabolic labeling during the 18 hr DEX+IL-6 treatment (Fig. 1B). The FBG secreted and accumulated in the medium over 18 hr showed equal labeling of Aα, Bβ and γ chains (i.e., equal relative specific activity) in accordance with their respective numbers of Met and Cys (not shown). After 18 hr treatment, cells were chased in label-free medium with DEX+IL-6 and the relative amount of FBG secreted in one hr increments up to 7 hr was determined after immunopurification, SDS-PAGE and fluorography (Fig. 1B). Complete labeling of intracellular FBG precursors during the 18 hr DEX+IL-6 treatment allowed us to examine the disappearance of each chain over time as the labeled precursors were assembled into whole FBG for secretion. After 1 hr, nonstimulated HepG2 and stimulated HepG2 and A549 cells still showed the expected ratio of completely radiolabeled Aα, Bβ and γ chains (Fig. 1B). After 2-3 hours, the relative amount of radioactivity in the Bβ chain markedly declined, and by 5-6 hours, it was barely detectable. FBG secreted between 5-6 hours consists of predominantly non-labeled Bβ chain, while still containing, albeit reduced, radioactive Aα and γ chains drawn from existing reservoirs (Fig. 1B, lanes 4-6). The radioactivity remained in the γ chain bands the longest, indicating that the free γ chain pool was the most abundant. By 6-7 hours, the radioactive pools of intracellular precursors were essentially depleted. The slower migrating γ chain results from alternative splicing of the γ chain pre-mRNA (25) and is evident in all treatment conditions of both HepG2 and A549 cells (Fig. 1B). Together, these results show that after 18 hours of DEX+IL-6 treatment, intracellular pools of free Aα and γ chain polypeptides exist in both cell types and that nascent synthesis of the Bβ is the rate-limiting step in regulated synthesis of FBG.

<table>
<thead>
<tr>
<th>Chase Time</th>
<th>Chain</th>
<th>HepG2-Control (n = 4)</th>
<th>HepG2-DEX+IL-6 (n = 4)</th>
<th>A549-DEX+IL-6 (n = 6)</th>
</tr>
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<tr>
<td>30 Min</td>
<td>Aα Chain</td>
<td>30.2 ± 3.1 *</td>
<td>31.5 ± 3.8</td>
<td>32.7 ± 6.7</td>
</tr>
<tr>
<td></td>
<td>Bβ Chain</td>
<td>49.1 ± 5.3</td>
<td>45.2 ± 5.8</td>
<td>46.5 ± 7.2</td>
</tr>
<tr>
<td></td>
<td>γ Chain</td>
<td>20.7 ± 4.8 **</td>
<td>23.3 ± 2.1 *</td>
<td>20.8 ± 1.9 **</td>
</tr>
<tr>
<td>60 Min</td>
<td>Aα Chain</td>
<td>31.0 ± 2.6 **</td>
<td>26.4 ± 5.5</td>
<td>27.4 ± 2.5 **</td>
</tr>
<tr>
<td></td>
<td>γ Chain</td>
<td>35.4 ± 5.6</td>
<td>28.6 ± 4.3</td>
<td>30.7 ± 3.0</td>
</tr>
<tr>
<td>90 Min</td>
<td>Bβ Chain</td>
<td>37.1 ± 6.9</td>
<td>44.0 ± 6.9</td>
<td>38.4 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>γ Chain</td>
<td>27.5 ± 3.3</td>
<td>27.4 ± 2.7</td>
<td>30.9 ± 1.7</td>
</tr>
</tbody>
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**Table 1: Percent of total secreted fibrinogen represented by each chain.**
Cells were treated for 18 hr in the absence or presence of DEX+IL-6 then the cells were pulse-labeled for 3 min with EasyTag Express Labeling Mix. Nascent FBG was immunopurified from the medium of each sample at 30-min intervals over a 90 min chase period in the appropriate chase medium without label. The FBG polypeptide chains were reduced and resolved by SDS-PAGE. The resulting fluorographs were scanned using NIH Image software and the labeling density of each chain was corrected for the relative number of Met and Cys in each chain. The data is presented for each polypeptide chain as the Percent of Total FBG secreted during that chase interval ± S.E.M.; *p<0.05 and **p<0.01 is relative to the amount of Bβ chain.
Relative rate of nascent synthesis of intracellular FBG

Previous studies have shown that the rates of synthesis of the FBG $\alpha\alpha$, $\beta\beta$ and $\gamma$ chains are unequal in hepatocytes (16). To determine whether the relative rate of synthesis of the individual polypeptide chains of FBG differed due to cell type and/or DEX+IL-6 treatment for 18 hr, such pretreated cells were pulse-labeled and the nascent intracellular chains were immunoprecipitated then analyzed by SDS-PAGE, fluorography, scanning densitometry and determination of statistical significance using StatView™. The data reveal that the relative rate of nascent synthesis of the $\beta\beta$ chain was greatest in control and treated HepG2 cells and that de novo synthesis of the $\alpha\alpha$ and $\gamma$ chains occurred at approximately the same rates (Fig. 2A). The increased rate of synthesis of the $\beta\beta$ chain compared to the $\alpha\alpha$ chains was statistically significant in control and treated HepG2 cells. In contrast, the rate of synthesis of all three chains in the DEX+IL-6-treated A549 cells was not significantly different. In the treated A549 cells, the $\alpha\alpha$ chain accounted for 32.7 ± 4.9%, the $\beta\beta$ chain for 29.5 ± 2.0% and the $\gamma$ chain for 37.8 ± 3.2% of the radioactivity (Fig. 2A). Furthermore, the relative rate of nascent $\beta\beta$ chain synthesis in response to DEX+IL-6 was significantly higher in HepG2 compared to A549 cells.

Intracellular precursor forms of FBG

To determine the fate of intracellular precursors of FBG, the disulfide-linked intermediates formed during a 3 min pulse (T=0) were examined after immunoprecipitation by 2% agarose slab gel electrophoresis under nonreducing conditions. The resulting fluorographs revealed that lower molecular weight precursors incorporate radioactivity within the 3 min pulse label in all conditions and into the FBG half-molecule ($\alpha\beta\gamma$) only in the DEX+IL-6-treated HepG2 cells (Fig. 3; T=0). The fate of intracellular precursors was followed at 10 min chase intervals for a total of 60 min. The band patterns were similar at all times for control and treated HepG2 cells with the exception that DEX+IL-6-treated HepG2 cells made significantly more FBG. FBG purified from stimulated A549 cells showed a different pattern of intracellular precursors. After a 3 min pulse and 10 min chase, all three individual polypeptides ($\alpha/\beta/\gamma$) were similarly labeled (Figs. 3 and 4), consistent with data shown in Figure 2. Intracellular two-chain intermediate precursors were similar to those in HepG2 cells. After 20 min, significantly more 340 kDa FBG ($[\alpha\beta\gamma]\beta\gamma$) was found cell-associated in A549 compared to HepG2 cells. In addition, DEX+IL-6 stimulation clearly upregulated the amount of each precursor molecule in the HepG2 cells, but did not increase the amount of intracellular FBG whole molecule.

The results presented in Figure 3 suggested that A549 cells accumulate whole FBG molecules ($[\alpha\beta\gamma]2$) prior to release into the medium. To determine whether nascent intact FBG accumulates with the cell-associated fraction in the lung epithelial cells, pulse-chase experiments were performed to follow both the intracellular accumulation of nascent FBG and the rate of secretion of radioactive FBG into the incubation medium (Fig. 3B and C). Control HepG2 and stimulated HepG2 and A549 cells were pulse-labeled for 3 min followed by chasing in label-free medium for the times indicated in the legend to Figure 3. Metabolically labeled FBG was immunopurified from the cell-associated (Cells) and secreted (Media) fractions of each sample and resolved under nonreducing conditions by SDS-PAGE. Immediately after the 3 min pulse-label (T=0), faintly labeled intact FBG was detected only in the cell-associated

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**Figure 2: Relative rate of nascent synthesis of intracellular FBG.** After 18 hr of treatment in the absence or presence of DEX+IL-6, HepG2 and A549 cell monolayers were pulse-labeled for 3 min followed by immediate washing and lysing of cells. The intracellular FBG chains were immunoprecipitated and analyzed by reducing and denaturing SDS-PAGE. Densitometric scans of the fluorographs from 3-4 separate experiments were analyzed and the intensity of each band was corrected for the number of Met and Cys to determine the labeling density of each chain as a percent of the total labeling density of the three chains (Percent of Total). Error bars represent ± standard error of the mean (S.E.M.). Statistical significance in the relative rate of synthesis of the individual chains in each treatment condition is indicated by a p-value < 0.05.
Figure 3: Fate of precursor forms of FBG. Panel A. Control HepG2 cells (left panel) and 18 hr DEX+IL-6-treated HepG2 (middle panel) and A549 (right panel) cells were pulse-labeled for 3 min, then chased for 10, 20, 40 or 60 min in label-free medium. Intracellular FBG precursor molecules were analyzed by 2% agarose slab gel electrophoresis under nonreducing conditions followed by fluorography. A representative fluorograph is shown (n=3). The positions of migration of the intracellular FBG precursor molecules and single chains are denoted to the right of the figure; the Mr markers are indicated to the left of the figure. Panels B and C. Control and treated HepG2 cells (panel B) and treated A549 cells (panel C) were pulse-labeled as described in panel A; the time intervals of chase in isotope-free medium is indicated above each panel. Immunopurified FBG, both cell-associated (Cells) and secreted into the medium (Media), was resolved by SDS-PAGE under nonreducing conditions. A representative fluorograph is shown.

Figure 4: Two-dimensional electrophoresis of intracellular precursor forms of FBG. The chain composition of the nascent FBG precursor molecules from lysed HepG2 cells (stimulated and control) and stimulated A549 cells were resolved in the first dimension under nonreducing conditions on 2% agarose slab gels (similar to gels shown in Fig 3A). Isolated polyacrylamide gel lanes were reduced and resolved in the second dimension by SDS-PAGE on 8% gels in Laemmli buffer (n=3). Cells pulse-labeled for 3 min were immediately lysed in a cocktail of detergents and protease inhibitors and designated time (T) T=0 (panel A) or cells were lysed after a 3 min pulse-label followed by a 60 min chase period and designated T=60 (panel B). A marker lane of reduced and denatured FBG polypeptide chains is shown for each panel.
fractions in each treatment group (Fig. 3B and C). In both control and DEX+IL-6-treated HepG2 cells, the labeling of intact FBG reached saturating intensity between 30-60 min in the cell-associated fractions, which also corresponds with the time of FBG secretion into the medium (Fig. 3B). After 60 min, secreted nascent FBG accumulated in media of both control and treated HepG2 cells, while remaining constant (but at relatively higher levels in the DEX+IL-6-treated cells) in the cell-assoc-
ated fractions. In contrast, nascent FBG accumulated in A549 cell-associated fractions reaching peak levels at 60 min (later times not shown); intact FBG released into medium began after 40 min of chase and continued to accumulate over time (Fig 3C). Earlier time points of nascent FBG produced by A549 cells are shown to emphasize the time-dependent accumulation of cell-associated FBG and delayed kinetics of release into the medium compared to HepG2 cells. These results confirm the data in Figure 3 which show accumulation of intact FBG rather than half-molecule in A549 but not HepG2 cells. Moreover, the results are consistent with our previous observations showing that nascent FBG secreted from A549 cells remains cell surface-associated prior to its release into the medium or assembly in the ECM (12).

Composition of nascent intracellular FBG precursors
The chain composition of precursor FBG molecules was analyzed by 2D-gel electrophoresis to facilitate understanding of the order of assembly of FBG chain intermediates in lung epithelial cells in response to upregulation by DEX+IL-6 (Fig. 4). At T=0, the results indicate that only FBG intermediates containing nascent radioactive Bβ chains were assembled in HepG2 control cells (Fig. 4A). In DEX+IL-6-treated HepG2 cells, intermediate chain precursors consisting of either Aα-γ or Bβ-γ chains were formed in addition to nascent Bβ and preformed Aα and γ chain-chain containing half-molecule (αβγ). A free pool of nascent γ chains was observed in DEX+IL-6-treated HepG2 cells, probably due to the hypersynthetic state induced by the 18 hr treatment. In contrast, at T=0 all three nascent chains (α/β/γ) were present in the cell-associated pool of treated A549 cells (Fig. 4A). The half-molecule (αβγ) containing nascent Bβ chain was also formed in the treated A549 cells (Fig. 4A). The faster migrating bands correspond to molecular weights of dimers of various Aα-γ or Bβ-γ two-chain intermediates and free Aα, Bβ, or γ chains. After 60 min of chase, HepG2 intracellular precursors were composed of significantly higher amounts of FBG half-molecule (αβγ) than whole molecule (αβγ2) whereas, the reverse was true for the A549 cells (Fig. 4B). Consistent with the data shown in Figures 2 and 3, A549 precursors were composed of significantly higher amounts of intact FBG (αβγ2) than half-molecule (αβγ) at T=60. Furthermore, all three chains labeled in nearly equal amounts (α/β/γ) were present in the cell-associated pool of the treated A549 but not control or treated HepG2 cells at T=60.

Analysis of N-linked glycosylation
Tunicamycin was used to determine whether N-linked glycosylation of the Bβ and γ chains occurs in A549 cells. FBG purified from the tunicamycin-treated cell lysates and culture medium clearly showed a more rapid migration of Bβ and γ chains by 7% Weber-Osborn SDS-PAGE, with the Aα chain remaining in the same position (Fig. 5). FBG synthesized by DEX+IL-6-treated lung epithelial cells is glycosylated on the Bβ and γ chains with the same apparent M, as shown previously in HepG2 cells (26). In addition, glycosylation of the Bβ and γ chains in HepG2 cells showed no changes in apparent Ms due to DEX+IL-6 treatment (Fig. 5).

Susceptibility to proteolysis
FBG was immunopurified from metabolically labeled DEX+IL-6-treated A549 cells was cleaved by thrombin (Fig. 6A) in a manner comparable to thrombin cleavage of plasma FBG (not shown) and FBG produced by control and DEX+IL-6-treated HepG2 cells (Fig. 6 A). The Aα and Bβ fibrin monomer chains showed faster migration than the Aα and Bβ chains consistent with the release of the FPA and FPB fragments, respectively (Fig. 6A). To confirm that thrombin cleavage results in exposure of the β-chain N-terminus, Western blotting showed that T2G1 recognizes the β 15–21 neoepitope in thrombin-treated samples only (Fig. 6B). Similarly, both HepG2 and A549 cell-derived FBG showed the same susceptibility to plasmin cleavage; the characteristic D and E fragments of FBG were generated (Fig. 6C).

Discussion
We show in this report that DEX+IL-6 regulated synthesis of FBG in hepatocytes follows the same pathway of assembly as previously shown for the constitutive synthesis of FBG by HepG2 cells (16, 22-24). Assembly commences by the independent attachment of the preformed Aα and γ chains, drawn from the intracellular pools, to the nascent Bβ chain. Two-chain complexes form, then each gains an additional chain from the precursor pools, forming the FBG half-molecule; two half-molecules are then assembled into dimeric FBG. The amount of HepG2 intracellular FBG whole molecule remained low, indicating that the intracellular whole molecule is transient and secreted soon after it is made. The Bβ chain synthesis remains the rate-limiting step in the hepatic cell production of FBG in response to DEX+IL-6 treatment.

Although other reports show that intestinal epithelial cells express FBG genes in response to IL-6 (11), this is the only report to demonstrate the pattern of FBG biosynthesis and assembly in response to DEX+IL-6 by extrahepatic epithelium. FBG protein produced by lung epithelium is essentially the same as that produced by hepatocytes with several notable exceptions. First, the rate of synthesis of each component chain was unequal in HepG2 cells, whereas the nascent synthesis of all three chains occurred at equivalent rates in A549 cells. This is consistent with data showing that in the absence of DEX+IL-6 treatment FBG is not produced constitutively by lung epithelial cells (8, 9, 12, 13). Instead, the biosynthesis of de novo FBG protein by lung epithelial cells occurs only by the DEX+IL-6
regulated pathway of FBG expression evoked during a pulmonary APR. However, after prolonged treatment of A549 cells with DEX+IL-6, the nascent Aα and γ chains begin to accumulate intracellularly, likely due to the more rapid degradation of the Bβ chain shown to occur in HepG2 cells (27). Therefore, once FBG biosynthesis is initiated, the kinetics of intermediate chain assembly of intact FBG by stimulated A549 cells resembles that of both nontreated and DEX+IL-6-treated HepG2 cells. Second, unlike the HepG2 cells that rapidly secreted fully formed FBG whole molecule, the DEX+IL-6-treated A549 cells contained a substantial amount of intact FBG in the intracellular pool, suggesting that the FBG is secreted at a slower rate from the lung epithelial cells than hepatocytes. Alternatively, the data suggest that newly secreted FBG binds to the A549 cell surface prior to release into the supernatant, thus contributing to a higher pool of cell-associated FBG. This is consistent with our previous results (12) showing that nascent FBG produced by A549 cells in response to DEX+IL-6 treatment binds to the cell surface in a saturable manner prior to its release into medium or deposition into ECM along with fibronectin in the detergent insoluble ECM fraction. Third, secretion of lung epithelial cell-derived FBG is polarized to the basolateral face and occurs only when the alveolar epithelial cell barrier integrity is maintained as shown by the presence of tight junctions; newly synthesized FBG is secreted in equivalent amounts from hepatocytes (13).

The soluble form of FBG secreted by lung alveolar epithelial cells is similar to hepatic cell FBG in terms of N-linked glycosylation of the Bβ and γ chains as well as susceptibility to both thrombin and plasmin cleavage. Thus, soluble FBG secreted from stimulated lung epithelium is susceptible to fibrin clot formation and dissolution, which would have adverse consequences in injured lung. Lung cells express elevated levels of IL-6 during acute or chronic inflammation (28, 29) and a growing body of evidence indicates that several APR proteins are produced in lung cells during inflammation. In addition to FBG, these proteins include haptoglobin (30), C-reactive protein (31), lipopolysaccharide binding protein (32), serum amyloid A (33), annexin I (34), and α1-antitrypsin (35). C-reactive protein and fibrinogen binding to lung surfactant contribute to abnormalities of surfactant function. Furthermore, persistent alveolar fibrinogen deposition is a morphological hallmark of severe or chronic lung injury (36). Thus, regulated synthesis, polarized secretion and deposition of FBG into the ECM during inflammation likely contribute to the maintenance of lung homeostasis. In contrast, once the alveolar epithelial barrier integrity is severely compromised, extravasation of plasma FBG and fibrin deposition in the lumen of the alveolar spaces would play a predominant role in disrupting surfactant homeostasis leading to lung dysfunction and perhaps fibrosis.

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