Enhancement of Procollagen Biosynthesis by p180 through Augmented Ribosome Association on the Endoplasmic Reticulum in Response to Stimulated Secretion

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A coiled-coil microtubule-bundling protein, p180, was originally reported as a ribosome-binding protein on the rough endoplasmic reticulum (ER) and is highly expressed in secretory tissues. Recently, we reported a novel role for p180 in the trans-Golgi network (TGN) expansion following stimulated collagen secretion. Here, we show that p180 plays a key role in procollagen biosynthesis and secretion in diploid fibroblasts. Depletion of p180 caused marked reductions of secreted collagens without significant loss of the ER membrane or mRNA. Metabolic labeling experiments revealed that the procollagen biosynthetic activity was markedly affected following p180 depletion. Moreover, loss of p180 perturbs ascorbate-stimulated de novo biosynthesis mainly in the membrane fraction with a preferential secretion defect of large proteins. At the EM level, one of the most prominent morphological features of p180-depleted cells was insufficient ribosome association on the ER membranes. In contrast, the ER of control cells was studded with numerous ribosomes, which were further enhanced by ascorbate. Similarly, biochemical analysis confirmed that levels of membrane-bound ribosomes were altered in a p180-dependent manner. Taken together, our data suggest that p180 plays crucial roles in enhancing collagen biosynthesis at the entry site of the secretory compartments by a novel mechanism that mainly involves facilitating ribosome association on the ER.

Entry of proteins into the secretory pathway via the rough endoplasmic reticulum (ER) is essential for intracellular transport of proteins to secretory compartments within the cell and finally to the extracellular milieu. In tissues with high secretory activity, such as the pancreas, the secretory apparatus is highly developed. One of the morphological hallmarks in such tissues is drastic proliferation of rough ER membranes that are densely occupied by ribosomes, whereas the rough ER in other tissues forms a loose network of tubular cisternae sparsely studded with ribosomes (1). However, the molecular basis for the biosynthesis and proliferation of the rough ER in secretory tissues is largely unknown (1, 2).

Collagen is one of the major components of the extracellular matrix (ECM) in connective tissues such as skin, tendon, and bone. It is synthesized on the ER membrane as a precursor form, i.e. procollagen, and secreted by professional secretory cells including fibroblasts. Fully consistent with the normal secretory pathway, procollagen is cotranslationally translated into the lumen of the ER. Much interest has been focused on the mechanisms of its folding and trimerization processes in the ER, such as the hydroxylation enzymes for proline and lysine residues (reviewed in Refs. 3 and 4)). Recently, there has been considerable interest in the intracellular trafficking mechanism of procollagen as a representative model for supramolecular cargos (5–7). In addition, the regulation of procollagen biosynthesis has been intensively studied at the transcriptional level (8, 9) as well as at the post-transcriptional level with a focus on mRNA stability (10, 11). After ascorbate stimulation, enhancement of collagen production mediated at the translational level has been suggested (12), but the molecular mechanism has remained elusive.

Ascorbate acts as a cofactor of prolylhydroxylase and promotes procollagen folding in the ER, thereby initiating its subsequent transport from the ER to the Golgi complex. Although many reports have utilized ascorbate as a stimulator for high-rate secretion (13) or as an inducer of synchronized procollagen trafficking (6, 7), the mechanism for the enhanced procollagen biosynthesis upon such stimulation has remained unclear. In response to increased traffic loads, regulated coordination of de novo biosynthesis must occur depending on the levels of undelivered cargos. A recent study elegantly showed that an increased protein load entering the cis-Golgi compartment from the ER activates the signaling circuit and coordinates the overall transport activity in the secretory pathway (14). However, little is known about the molecular mechanism for the enhanced de novo biosynthesis in response to high traffic loads.

p180 is an integral ER membrane protein that contains several functional domains including a predicted transmembrane domain, a tandem repeat domain involved in ribosome binding (15), and an MTB-1 domain responsible for microtubule binding and bundling (16). Based on overexpression experiments in yeast cells, which lack p180 protein, potential functions for p180 in the secretory process have been proposed that involve...
stabilization of mRNAs and ER membrane proliferation (17, 18). A recent study further addressed the roles of p180 in macrophage-like differentiation, and clearly demonstrated a key function for p180 in acquiring the secretory phenotype (19). Contrary to this report, p180 overexpression in a pancreatic islet β-cell line failed to enhance amylase secretion despite increased ER membranes (20), indicating the still undefined functions for p180 in the secretory process.

Recently, we demonstrated that high levels of p180 expression were required to develop the remarkably expanded morphology of the trans-Golgi network (TGN) in diploid fibroblasts, which permits efficient formation and transport of large post-Golgi carriers containing procollagen aggregates (21). During that study, we noticed that collagen secretion was impaired in p180-depleted cells. The aims of the present study were to clarify the roles of p180 in the regulatory mechanism of collagen secretion and how it contributes to the enhancement of the secretory capacity in professional collagen secreting cells. Using human embryonic lung (HEL) diploid fibroblasts, which contain abundant endogenous p180 (22) and possess a high capacity to secrete collagen (23), we demonstrate that human p180 plays a crucial role in the enhanced biosynthesis of procollagen by a novel mechanism probably at the translational level.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Short Interfering RNA (siRNA) Oligonucleotide Transfection**—HEL fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Nissui) supplemented with 10% fetal bovine serum (FBS) (Intergen). L-Ascorbic acid phosphate-magnesium salt 2-hydrate (Wako) was added to a final concentration of 0.2 mM unless otherwise indicated. Transfection of a siRNA targeting p180 was carried out essentially as described previously (16). siRNA duplexes (21 nucleotides) against human p180 (DDB) accession number AB287347) corresponding to nucleotides 153–173 (p180-1) were used unless otherwise indicated. In some experiments, siRNA duplexes against human p180 of corresponding nucleotides 271–291 (p180-2) were used. The protocols for cell culture and transfection are summarized in supplemental Fig. S1A.

Briefly, 1 × 10⁶ cells/well were seeded in 6-well plates precoated with fibronectin and incubated in DMEM containing 10% FBS for 9 h before transfection. Transfected cells were incubated in DMEM/F-12 medium for 12 h. After transfection, the medium was replaced with DMEM/F12 containing 2% FBS and subjected to the following analyses at 3–5 days post-transfection. A stable HeLa cell line overexpressing p180 (HeLa/p180) was described previously (21).

**Analysis of Collagens Secreted from Cultured Cells**—Collagens secreted into the culture medium during post-transfection days 3–5 were prepared as previously described (24). Briefly, culture medium was treated with pepsin (0.5 mg/ml in 0.1 N HCl) at 4 °C for 24 h. Cold ethanol was then added to a final concentration of 33% and incubated for 1 h on ice to precipitate the pepsin-solubilized collagens. After centrifugation, the obtained pellet was washed with 70% ethanol, resuspended in 0.1 N HCl, and lyophilized. The final pellet was solubilized in Laemmli SDS sample buffer, and subjected to SDS-PAGE under non-reducing conditions. The gel was stained with Coomassie Brilliant Blue R-250, and the relative quantities of the stained bands were estimated by densitometry using NIH Image.

**Antibodies, Reagents, and Western Blot Analysis**—An affinity-purified rabbit antibody against human p180 (N1) was described previously (22). The following antibodies were purchased: rabbit antibodies against calnexin and protein-disulfide isomerase (Stressgen), type I collagen (LB-1190,LSL), type VI collagen (Polysciences), and ribosomal protein L10 (Santa Cruz Biotechnology), biotin (Bethyl Lab), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling), mouse monoclonal antibodies against p115 and fibronectin (Transduction Laboratories), β-actin (Sigma), TIMP-1 (Chemicon), and human type I procollagen amino terminus (SP1.D8; Developmental Studies Hybridoma Bank, University of Iowa). Western blot analyses of p180 were performed as described previously (22). The samples were normalized by the DNA amounts. Recombinant human TIMP-1 (Daichi Fine Chemical Co., Japan) was used as control. Protease inhibitors (EDTA-free Complete Inhibitor Mixture) were from Roche Applied Science.

**Quantification of mRNAs by Real Time PCR**—Total RNA was isolated from cells using an SV Total RNA Isolation System (Promega). cDNA was synthesized using a TaqMan® Gold RT-PCR Kit (Applied Biosystems) with random hexamer primers. Quantitative real time PCR was performed using a TaqMan Reverse Transcription Reagent (Applied Biosystems) in an ABI Prism 7300 Sequence Detection System (Applied Biosystems). Data were normalized by the expression of human MT-ATP6. The following gene-specific primers and probes were obtained from Applied Biosystems: human p180 (GenBank NM004587.2), human collagen type I α1 chain (GenBank NM000088.2), human fibronectin (GenBank NM212474.1), human collagen type VI α1 chain (GenBank NM001848.2), human tissue inhibitor of metalloproteinases (TIMP)-1 (GenBank NM003254.2), human matrix metalloproteinase (MMP)-2 (GenBank NM004530.2), and human MT-ATP6 (GenBank NC001807.4).

**Immunofluorescence Analysis**—Cells grown on coverslips were fixed with paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with antibodies as described previously (16). The cells were imaged using an FV1000 confocal microscope system (Olympus) with a ×40 lens (1.3 NA) or a ×20 lens (1.0 NA). Laser scanning microscopic images were sequentially obtained at 1-μm intervals and shown as deconvoluted images, because the collagen fibers formed on the cells with variable thicknesses in the Z-axis direction.

**Metabolic Radiolabeling of Cell Cultures and Analysis of Collagen**—Cells (1.5 × 10⁵) plated on fibronectin-coated 6-well dishes were transfected with siRNA as described above. After 3 days of incubation, the medium was changed to 1.0 ml of fresh DMEM containing 0.1% FBS, [3H]proline (100 μCi/ml; PerkinElmer Life Sciences), and 0.5 mM β-aminopropionitrile and incubated for another 20 h. Pepsin-solubilized collagens were prepared from the medium as described above, and examined by SDS-PAGE using a 5% gel, followed by fluorescence autoradiography. Alternatively, the total and collagenous protein biosynthetic activities were quantified using bacterial collagenase as previously described (25). Briefly, cell and medium fractions were treated
with bacterial collagenase at 37 °C for 16 h, and then precipitated by adding tannic acid and trichloroacetic acid at final concentrations of 0.5 and 10%, respectively. The resulting supernatants included oligopeptide fragments from [3H]proline-labeled collagen digested by bacterial collagenase, whereas collagenase-resistant proteins were precipitated. The radioactivities of the supernatant (collagenous proteins) and precipitate (non-collagenous proteins) fractions were measured using a liquid scintillation counter. The value for non-collagenous protein synthesis was calculated by assuming that collagen has a 5.4-fold higher amino acid content than other proteins (26).

Real Time Zymography—Real time zymography was performed as previously described (27). Briefly, fibroblasts were cultured in serum-free medium for 3 days, and the culture medium was collected. The supernatant was subjected to SDS-PAGE under non-reducing conditions using a 10% gel containing 0.05% FITC-labeled gelatin. The gel was washed in 50 mM Tris-HCl (pH 7.4) containing 2.5% Triton X-100 for 30 min, and incubated in 50 mM Tris-HCl (pH 7.4) containing 5 mM CaCl₂ and 150 mM NaCl at 37 °C for 16 h. The FITC-labeled gelatin digested by MMP-2 was visualized using a transilluminator. Human rheumatoid synovial fibroblast MMP-2 proenzyme (Calbiochem) was used as control.

Electron Microscopy (EM)—For transmission EM analysis, cells cultured in 0.1% FBS/DMEM were treated with medium containing 10 μM taxol for 3 min at 37 °C, followed by a conventional fixation procedure for transmission EM as described previously (22). Ultrathin sections were cut parallel to the substrate and viewed under a model 7650 EM (Hitachi) at 80 kV.

Sequential Detergent Extractions of the Membrane Fraction—Cytosolic and membrane fractions were obtained by sequential detergent extractions as described previously (28) with the following modifications. Briefly, HEL cell monolayers in 6-well plates were precultured in the presence of 200 μM HEPES, pH 7.5, 2.5 mM Mg(OAc)₂, 1 mM EGTA, 0.004% digitonin, protease inhibitor mixture. The membrane fraction was then extracted with 0.2 ml of lysis buffer (400 mM KOAc, 25 mM HEPES, pH 7.5, 15 mM Mg(OAc)₂, 1 mM EGTA, 1% Nonidet P-40, 0.5% deoxycholate, protease inhibitor mixture) for 10 min on ice. The solution containing the membrane fraction was carefully collected and cleared by centrifugation at 7500 × g for 10 min at 4 °C to remove insoluble debris. The resulting supernatant was subjected to Western blotting analyses. Detergent extractions of HeLa cells were performed using permeabilization buffer containing 0.05% digitonin for 10 min on ice, followed by the same procedure as described above.

Non-radioisotopic Labeling of Newly Synthesized Proteins with Avidin-Digoxigenin (AHA) and Conjugation with Biotin-alkyne by Click Reaction—To estimate activity of de novo protein biosynthesis in membrane fractions, we used the non-radioisotopic labeling system for newly synthesized proteins with AHA, which serves as a surrogate for methionine (29). After 3 days post siRNA transfection, the medium was replaced with 1-methionine-free DMEM and precultured for 1 h. AHA (final concentration, 50 μM) was added to the medium and cultured for another 4 h. Then cells were subjected to sequential detergent extraction to obtain membrane and cytosolic fractions. Each sample was precipitated with trichloroacetic acid, and then the click reaction was performed to conjugate incorporated AHA with biotin containing alkyne in the click reaction buffer (Invitrogen) according to the manufacturer’s instructions. Samples were precipitated by trichloroacetic acid, followed by blotting analyses using anti-biotin antibody.

To estimate newly synthesized proteins secreted into culture medium by pulse label experiments, cells preincubated with 1-methionine-free medium for 1 h were cultured for 4 h in the presence of AHA (final concentration, 50 μM). Then cells were extensively washed with PBS, and the culture medium was replaced with fresh DMEM containing 0.1% FBS. After 18 h culture, medium was harvested and precipitated at 10% trichloroacetic acid. Conjugation of incorporated AHA with biotin and blotting analyses were carried out as described above. Specificity of metabolic labeling of newly synthesized proteins with AHA was assessed by comparing biotin-tagged protein patterns in the presence and absence of cycloheximide, or those in cultured with methionine instead of AHA (supplemental Fig. S3).

RESULTS

p180 Knockdown Suppresses Collagen Secretion—To explore the roles of p180 in the secretory function, we manipulated the expression levels of p180 in HEL cells by siRNA (p180-1) transfection and examined the effects on collagen secretion. First, we confirmed effective suppression of p180 mRNA levels at 3 or 4 days post-transfection, and found that they were suppressed to <35% of the levels in control cells (data not shown). Strong suppression of p180 protein was confirmed by Western blotting analyses, whereas the expression levels of other endogenous ER markers including calnexin and protein-disulfide isomerase remained unchanged (Supplemental Fig. S1, B and C), as reported previously (16, 21). Next, we addressed whether p180 depletion affects collagen secretion. HEL cells grown in the presence of ascorbate secreted a large amount of collagen into the culture medium, including type I and type III collagens (Fig. 1A) (30). In contrast, the secretion of type I collagen in p180-depleted cells was suppressed to less than one-half the levels in control cells (by 57 and 66% for the α1 and α2 chains, respectively). Likewise, reduced secretion of type III collagen was observed following p180 depletion (Fig. 1A). Consistently, immunofluorescence analyses revealed that p180-depleted cells exhibited immature deposits of type I collagen fibrils on the cells and in the extracellular space, whereas well organized deposits were visible for control cells (Fig. 1B). To exclude possible off-target effects of the siRNA (p180-1), we examined additional siRNA experiments using another p180 sequence (p180-2). Knockdown of p180 by siRNA (p180-2) also induced prominent reduction of secreted collagen as shown in supplemental Fig. S2A. Thus, the loss of p180 appears to disturb
efficient collagen secretion into the extracellular milieu in these fibroblasts.

**Loss of p180 Results in Decreased Activity of Procollagen Biosynthesis**—Collagen is synthesized as a precursor form, *i.e.*, procollagen, the biosynthesis of which begins on the rough ER membrane, followed by a series of modification and folding steps in the ER and Golgi compartments. To determine whether its biosynthesis is perturbed in the absence of p180, the protein levels of intracellular procollagen were analyzed. Western blotting analyses revealed that p180 depletion caused a marked reduction of the intracellular procollagen levels, which were suppressed to about 50% of the levels in control cells (Fig. 1C), implying reduced biosynthetic activity.

Next, we performed metabolic labeling experiments to estimate the biosynthetic activity of the p180-depleted cells in more detail. After [3H]proline was incorporated into HEL cells, pepsin-solubilized collagens were prepared from the medium and cell layer, and analyzed by SDS-PAGE. Fluorograms showed clear reductions in newly synthesized collagen in both the cell layer and medium fractions of p180-depleted cells (Fig. 2). Measurement of [3H]proline incorporation into the total protein fractions revealed that loss of p180 markedly suppressed the total protein biosynthesis by about 40% (Table 1). Interestingly, the biosynthesis of collagenous proteins (*i.e.* collagenase-digestible fraction) was more severely perturbed in p180-depleted cells, in which it was decreased by 60%. Therefore, loss of endogenous p180 leads to overall suppression of protein biosynthesis, and this effect is more notable on procollagen biosynthesis.
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mRNA Levels of Secreted Proteins Remain Unchanged following p180 Knockdown—To determine whether the suppression of protein biosynthesis was caused by reduced mRNA levels, we quantified the mRNA levels for several secretory proteins including types I and VI collagens, fibronectin, TIMP-1, and MMP-2 (Fig. 3A). In addition, the mRNA levels of other cellular proteins including nuclear, membranous, mitochondrial, and cytosolic proteins were also determined (data not shown). p180 depletion resulted in no significant reduction of mRNAs of the examined proteins except p180, whereas levels of collagen and fibronectin mRNAs showed relatively large variations (Fig. 3A). Therefore the reduced activity of protein biosynthesis probably occurs at post-transcriptional steps.

Loss of p180 Leads to Impaired Association of Ribosomes on the ER—It is well known that fibroblasts grown in the presence of ascorbate exhibit a highly developed morphology of the ER and Golgi complex, as reported in previous studies (31, 32) and also shown in our recent study (21). The reduced activity of protein biosynthesis after p180 suppression may represent aberrant organization of the secretory compartments as a consequence of general cellular damage. However, we previously found that p180 suppression causes neither disorganized ultrastructures of the ER and Golgi complex nor dysfunction of procollagen trafficking, although cells expressing low levels of p180 fail to exhibit well developed Golgi phenotypes including TGN expansion and peripheral dilated distensions (21). Further inspections and comparisons of EM images between p180-depleted and control cells revealed that one of the most prominent features of control cells was a large amount of ribosomes on the ER (Fig. 4A). A higher magnification image showed densely studded ribosome arrays on the ER membranes (Fig. 4B). In contrast, p180-depleted cells exhibited apparently reduced studding of ribosomes on the ER membranes (Fig. 4, C–E), without apparent disturbance of the rough ER morphology.

Expression Levels of p180 Are Well Correlated with Membrane-associated Ribosomes—To examine the ribosome contents associated with the ER membranes, we prepared membrane and cytosolic fractions by sequential detergent extractions using digitonin as described previously (28). Marker protein analyses confirmed the expected enrichment of membrane and cytosolic proteins in each fraction (Fig. 5, A and B). When the protein levels of ribosomal protein L10 (RPL10) were compared between the membrane-bound and cytosolic fractions, it was evident that more than 80% of the RPL10 was present in the membrane fractions in HEL cells (Fig. 5, A and B), which is remarkably higher than previously reported data for HeLa cells (33). Then we compared expression levels of p180 and RPL10 among cells cultured in the presence and absence of ascorbate. Ascorbate stimulation resulted in large increases in endogenous p180 expression by greater than 2-fold (Fig. 5A). Concomitantly, membrane-bound RPL10 was elevated by

| TABLE 1 |
| Production of [3H]proline-labeled collagen and non-collagenous proteins from HEL fibroblasts |

| Cell          | Collagen (A) | Non-collagenous protein (B) | Collagen/total protein |
|---------------|--------------|-----------------------------|------------------------|
| Collagen (A)  | 92.0 ± 3.6   | 546.6 ± 5.2                 | 14.4 ± 0.5             |
| Non-collagenous protein (B) | 36.8 ± 6.2 (0.400) | 343.3 ± 8.2 (0.628) | 9.6 ± 1.3             |

Data represent mean ± S.D. of quadruplicate assays.

| Collagen (A) | Non-collagenous protein (B) | Collagen/total protein |
|--------------|-----------------------------|------------------------|
| Mock         | 92.0 ± 3.6                  | 14.4 ± 0.5             |
| siRNA        | 36.8 ± 6.2 (0.400)          | 9.6 ± 1.3             |

Data in parentheses represent the ratio of [3H]proline-labeled proteins produced by siRNA-treated cells to those by mock cells.

Figure 3. Relative mRNA levels of various secretory proteins after p180 depletion. mRNA levels were estimated by real time PCR following treatment with a p180-specific siRNA (p180-1) (A). Relative mRNA levels of total cells. Data were normalized by the amounts of MT-ATP6 mRNA. The data represent the mean ± S.D. of three separate experiments, and are indicated as percentages of the corresponding levels in control cells set at 100% (dotted line). Data for type I collagen (col I), type VI collagen (col VI), fibronectin (FN), TIMP-1, and MMP-2 are shown. B, cytosolic and membrane fractions of HEL cells treated by ascorbate and/or siRNA (p180-1) were prepared by sequential digitonin treatment. Relative mRNA levels of cytosolic or membrane fractions are shown for type I collagen (COL IA1), fibronectin, and TIMP-1. Cyto, cytosol; mem, membrane.
Depletion of Endogenous p180 Perturbs Ascorbate-stimulated de Novo Biosynthesis in Membrane Fraction—To estimate activity of de novo biosynthesis in membrane fractions, we utilized the non-radioisotopic labeling system with AHA (29), which is specifically incorporated into newly synthesized proteins as a surrogate for methionine (29). First we verified specific and efficient incorporation of AHA into newly synthesized proteins in HEL cells, and confirmed that newly synthesized proteins can be detected specifically (supplemental Fig. S3). Then we examined the general activity of de novo biosynthesis following ascorbate treatment and p180 depletion. Ascorbate treatment induced prominent elevation of biosynthesis in total cell lysates (Fig. 6A, lane 2). When membrane and cytosolic fractions were compared, stimulatory effects were found mainly in the membrane fraction (lane 8), consistent with increased ribosomes in this fraction (Fig. 5A).

Furthermore, the activation was remarkably perturbed by loss of p180 (Fig. 6A, lane 3 and 9), similar to the data by [3H]proline labeling (Table 1). Among these AHA-incorporated proteins, suppression caused by p180 depletion seemed to occur not uniformly, indicating a preferential effect on some proteins versus others. In cytosolic fractions, in contrast, activity of de novo biosynthesis was kept at low levels regardless of ascorbate treatment or p180 depletion (lanes 4−6).

Next we studied the general secretory activity of ascorbate-stimulated and p180-depleted cells. AHA-labeled proteins secreted into medium were detected after washout and 18 h culture (Fig. 6B, lanes 1−3), whereas control cells treated by methionine failed to show any signals (lanes 4−6). Consistent with elevated biosynthesis in the membrane fractions upon stimulation (Fig. 6A), higher signals were found in the medium fraction of ascorbate-stimulated cells as compared with that of non-stimulated cells (Fig. 6B, lane 1 and 2). Again p180-knockdown perturbed secretion of some proteins, especially those with higher than 150 kDa, whereas secretion of smaller proteins was minimally affected (lane 3).
Depletion of Endogenous p180 Preferentially Suppresses Secretion of ECM Proteins—To address whether p180 suppression can induce preferential defects in secretion of some macromolecules, two classes of proteins were examined, namely ECM proteins and smaller proteins including TIMP-1 and MMP-2. Proteins from both classes are actively secreted from HEL fibroblasts. Immunostaining for type VI collagen and fibronectin revealed that p180-depleted cells exhibited much less deposition of these ECM proteins than control cells (Fig. 7A), similar to the data for type I collagen (Fig. 1B). In contrast, secretion of TIMP-1 and MMP-2 were hardly changed by the low levels of p180 (Fig. 7B and supplemental Fig. S2C), suggesting that the general biosynthesis and trafficking of secretory proteins are not markedly abolished.

Depletion of Endogenous p180 Preferentially Suppresses Secretion of ECM Proteins—To address whether p180 suppression can induce preferential defects in secretion of some macromolecules, two classes of proteins were examined, namely ECM proteins and smaller proteins including TIMP-1 and MMP-2. Proteins from both classes are actively secreted from HEL fibroblasts. Immunostaining for type VI collagen and fibronectin revealed that p180-depleted cells exhibited much less deposition of these ECM proteins than control cells (Fig. 7A), similar to the data for type I collagen (Fig. 1B). In contrast, secretion of TIMP-1 and MMP-2 were hardly changed by the low levels of p180 (Fig. 7B and supplemental Fig. S2C), suggesting that the general biosynthesis and trafficking of secretory proteins are not markedly abolished.

Next we studied whether manipulation of p180 affects mRNA levels in membrane fractions. As shown in Fig. 3B, mRNAs of type I collagen and fibronectin are mostly detected in membrane fractions. Following ascorbate stimulation or p180 depletion, levels of these mRNAs in membrane fractions remained unchanged, although a slight, not significant, decrease for type I collagen and fibronectin was seen (Fig. 3B). It is note-
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**FIGURE 8.** Manipulation of p180 expression levels affects the amount of membrane-associated ribosomes in HeLa transfectants. The amounts of ER-associated and cytosolic ribosomes were compared among stable HeLa transfectants overexpressing p180 (HeLa/p180) (lanes 2, 3, 5, and 6) and control cells (HeLa/puro) (lanes 1 and 4). Membrane and cytosolic fractions were prepared by sequential detergent extraction from p180-depleted (lanes 3 and 6) and untreated (lanes 2 and 5) HeLa/p180 cells and HeLa/puro cells (lanes 1 and 4), as described in the legend for Fig. 5. A, ribosomal RNAs were analyzed by agarose gel electrophoresis and stained with ethidium bromide. B, membrane and cytosolic fractions were analyzed by immunoblotting. C, EM images of HeLa/puro (a and c) and HeLa/p180 (b and d) cells. Ribosomes studded on the membranes are shown by arrows. Bars: a and b, 500 nm; c and d, 100 nm. Cnx, calnexin.

**DISCUSSION**

The molecular mechanism for the enhanced *de novo* biosynthesis of procollagen upon ascorbate stimulation has remained elusive (12, 34, 35). The present study provides evidence that p180 plays a key role in the highly efficient biosynthesis of type I procollagen and its subsequent secretion into the extracellular space. We also found that p180 depletion causes reduced activity of protein biosynthesis on the ER, without significant reduction of the membranes or mRNAs. Reversely, elevation of the p180 levels by ascorbate stimulation or overexpression resulted in a concomitant increase in membrane-bound ribosomes. These data suggest that p180 is directly relevant to the ribosome association with the ER membranes. This novel ability of p180 probably permits high-rate protein synthesis during activated collagen secretion in professional collagen secretory cells. The involvement of p180 in ribosome association on the ER is in accordance with the original study that identified p180 as a ribosome-binding protein by cross-linking experiments (36). However, it still remains obscure whether ribosome association with the ER is mediated by direct binding to p180 protein or by modulating other factors. Further study is necessary to elucidate a role for p180 in ribosome association with the ER in more detail, including *in vitro* binding experiments using recombinant proteins.

Recently, broad functions for ER-bound ribosomes have been demonstrated. Genome-wide studies examining mRNA populations on cytosolic and ER-bound polysomes have revealed an unexpected overlap between the two mRNA pools in eukaryotic cells (37, 38), and a significant fraction of cytosolic proteins undergo synthesis on ER-bound ribosomes (33). Moreover, ER-associated ribosomes have been shown to mediate more efficient biosynthesis than free ribosomes (33). From this point of view, further study on the molecular basis underlying preferential effects on large molecules by p180 may provide insights into the general regulation of protein biosynthesis.

It is noteworthy that there are two classes of membrane-associated ribosomes, namely p180-dependent and p180-independent association, in HEL cells (Fig. 5) and in p180-overexpressing HeLa transfectants (Fig. 8). Our data also demonstrate that the p180-dependent activation of collagen biosynthesis occurs without further recruitment of mRNA to the membrane, suggesting availability of ribosomes on the ER is important rather than mRNA amounts. A novel regulatory mechanism may exist that modulates the translation rate in actively procollagen producing cells. Such a mechanism at the translational level has been predicted for collagen-secreting fibro-
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Recent proteome studies have shown that p180 is involved in a novel mechanism for efficient translation for this class of proteins. The role of p180 in the regulation of protein biosynthesis is supported by our following investigations. Moreover, our findings are consistent with previous reports indicating that increased p180 levels can induce rough ER membrane proliferation. The above mentioned studies indicate that elevated p180 protein levels are accompanied by increased production of rough ER as the common output data, our findings are distinct from these reports regarding ER membrane proliferation. In contrast, our data for HEL fibroblasts demonstrate that the change in the ER membrane amounts was minimal when p180 was either up-regulated upon ascorbate stimulation or depleted by siRNA targeting, as reported recently (16, 21). Instead, we found that the biosynthetic activity for procollagens was reduced without significant loss of mRNAs or perturbation of the ER to Golgi traffic, again in contrast to data for THP-1 cells, which showed impaired apoe secretion because of aberrant intracellular trafficking upon p180 depletion (19).

One of the possible reasons for these discrepancies is the diverse stages of the differentiation of the cells used in these studies. Fibroblasts are terminally differentiated cells that are dedicated to producing copious amounts of collagen, whereas THP-1 cells are not differentiated but remain differentiation-competent. In terminally differentiated fibroblasts, p180 probably plays a key role in the activation process to respond to expanded trafficking demands after stimulation, whereas the maintenance of the ER membrane seems to be independent of the p180 protein levels. This idea is supported by our following findings. First, HEL cells expressing low levels of p180 were still able to secrete basal levels of procollagen (Fig. 1) and retain normal functions for procollagen transport out of the ER (21), but could not accommodate high rate synthesis of procollagen (Fig. 2 and Table 1). Second, based on EM imaging and Western blotting analyses, knockdown of p180 failed to disrupt the normal ER organization, and only affected the ribosome density on ER membranes without significant loss of the membrane amounts (Fig. 4 and supplemental Fig. S1).

Taken together, p180 seems to be involved primarily in high-rate protein biogenesis at the entry site of the secretory compartments, as well as in maintaining the unique TGN morphology (21). Subsequently, more efficient up-regulation of protein biogenesis probably occurs through a further augmentation of the ribosome association with the ER in response to high traffic loads. Such a cycling process of higher traffic loads and activated biosynthesis may be coordinately coupled if the cells contain high levels of p180. From this point of view, p180 may play a crucial role in the regulated coordination of multiple secretory compartments to provide an advantageous platform especially for the biogenesis and secretion of ECM proteins.

The molecular mechanisms for rough ER biogenesis and compartmentalization are largely unknown (1, 2). Further investigations on the interplay between p180 and the ribosome machinery may provide important insights into the mechanism of coordinated regulation of the highly efficient secretion achieved by professional secretory cells.

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