Knockdown of p180 Eliminates the Terminal Differentiation of a Secretory Cell Line

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We have previously reported that the expression in yeast of an integral membrane protein (p180) of the endoplasmic reticulum (ER), isolated for its ability to mediate ribosome binding, is capable of inducing new membrane biogenesis and an increase in secretory capacity. To demonstrate that p180 is necessary and sufficient for terminal differentiation and acquisition of a secretory phenotype in mammalian cells, we studied the differentiation of a secretory cell line where p180 levels had been significantly reduced using RNAi technology and by transiently expressing p180 in nonsecretory cells. A human monocytic (THP-1) cell line, that can acquire macrophage-like properties, failed to proliferate rough ER when p180 levels were lowered. The Golgi compartment and the secretion of apolipoprotein E (Apo E) were dramatically affected in cells expressing reduced p180 levels. On the other hand, expression of p180 in a human embryonic kidney nonsecretory cell line (HEK293) showed a significant increase in proliferation of rough ER membranes and Golgi complexes. The results obtained from knockdown and overexpression experiments demonstrate that p180 is both necessary and sufficient to induce a secretory phenotype in mammalian cells. These findings support a central role for p180 in the terminal differentiation of secretory cells and tissues.

INTRODUCTION

At the morphological level, the development of secretory cells and tissues has been well documented. From a molecular point of view, details of how cells acquire the ability to secrete at high levels as part of the process of their terminal differentiation are less well understood. Early in mammalian embryogenesis, the cytoplasm of cells of exocrine tissues, such as pancreas or liver, possesses a mere fraction of the intracellular organelles that they ultimately acquire upon terminal differentiation (Dallner *et al.*, 1966a,b; Pictet *et al.*, 1972; Slack, 1995; Debas, 1997). As differentiation of these tissues progresses, their cells take on a morphology that is dominated by membranous elements of the secretory pathway, most notably rough endoplasmic reticulum (ER) (Jamieson and Palade, 1967a,b). A similar situation exists in the immune system, in which B-lymphocytes differentiate into plasma cells as the result of being triggered by the appropriate antigen (Janossy *et al.*, 1973; Shohat *et al.*, 1973; Wiest *et al.*, 1989, 1990; Chen-Kiang 1995; Gass *et al.*, 2002; Iwakoshi *et al.*, 2003; Federovitch *et al.*, 2005; Fagone *et al.*, 2007). A prerequisite for achieving such a “secretory phenotype” is intracellular membrane proliferation, which is in turn needed to expand the cell’s capacity to synthesize, process, transport, and ultimately release peptides and proteins into the extracellular space.

Limited success has been achieved, in a few cell types, in recapitulating membrane proliferation reminiscent of secretory cell differentiation. Through the ectopic expression of membrane proteins, ER-like membranes have been produced in yeast as well as in various mammalian cells (Wright *et al.*, 1988; Vergeres *et al.*, 1993; Takei *et al.*, 1994; Vergeres *et al.*, 1995; Koning *et al.*, 1996; Cox *et al.*, 1997; Menzel *et al.*, 1997; Kim *et al.*, 2000; Sriburi *et al.*, 2004; Bai *et al.*, 2007). Although the extent of membrane proliferation as well as the individual morphologies vary greatly, only one such system has yielded functional membranes whose proliferation coincided with increases in a cell’s secretory capacity. This was achieved when a mammalian ribosome binding protein from the rough ER (p180) was expressed in yeast cells (Savitz and Meyer, 1990; Wanker *et al.*, 1995; Becker *et al.*, 1999). Proliferation of functional ER membranes was accompanied by a four- to fivefold increase in the secretion of a coexpressed protein into the medium (Becker *et al.*, 1999).

In yeast, the expression of p180 was the only stimulus needed to trigger secretory pathway biogenesis and increased levels of secretion (Becker *et al.*, 1999). Originally isolated using a functional assay for ribosome binding, p180 has several distinctive features (Savitz and Meyer, 1990; Wanker *et al.*, 1995). Anchored to the rough ER membrane by an N-terminal signal-anchor sequence of 28 amino acids, the remainder of p180 resides completely in the cytosol (Wanker *et al.*, 1995). It has high affinity ($K_d = 10\text{--}20\text{ nm}$) for ribosomes (Savitz and Meyer, 1990), mediated by a domain in which a 10-amino acid consensus motif (NQGKKAEGAP) is repeated 54 times without interruption (Wanker *et al.*, 1995). This domain is also needed for stabilization of mRNAs that encode proteins directed to the secretory pathway (Hyde *et al.*, 2002). The C-terminal half is very acidic ($pI = 4.99$) and predicted to form numerous coiled coils (Wanker *et al.*, 1995; Leung *et al.*, 1996; Diefenbach *et al.*, 2004; Ogawa-

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Abbreviations used: ER, endoplasmic reticulum; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; THP-1, cell line derived from human acute monocytic leukemia; shRNA, small-hairpin RNA.

Goto *et al.*, 2007). At a minimum, it is the membrane anchor and the ribosome binding domain that confer upon p180 its ability to produce functional rough ER membranes and concurrently increase the secretory capacity of these cells (Becker *et al.*, 1999; Hyde *et al.*, 2002; Bai *et al.*, 2007).

The bulk of studies on induced membrane proliferation have been carried out in yeast. Even those carried out in mammalian cells fail to ascertain physiological relevance for any of the ectopically expressed membrane proteins in the induction process. Genomic studies indicate that p180 may be restricted in its distribution to vertebrates; there is no orthologue in yeast (Wanker *et al.*, 1995; Leung *et al.*, 1996; Langley *et al.*, 1998; Kim *et al.*, 2000; Ogawa-Goto *et al.*, 2002, 2007). Other inducers of membrane biogenesis may have a wider species distribution, but they have not been found to stimulate the production of functional membranes (Wright *et al.*, 1988; Vergeres *et al.*, 1993; Takei *et al.*, 1994; Koning *et al.*, 1996; Cox *et al.*, 1997; Menzel *et al.*, 1997; Kim *et al.*, 2000; Sriburi *et al.*, 2004). If p180 plays a central role in the differentiation of a secretory cell, its reduced or eliminated expression should have severe consequences on the cell's ability to acquire a secretory phenotype.

A suitable test system is the THP-1 cell line. Of human monocytic origin, this line can be grown in culture and will acquire a macrophage-like secretory phenotype within 48–72 h after being stimulated by phorbol esters (Tsuchiya *et al.*, 1982; Hass, 1992, 1997). Some of the morphological and biochemical hallmarks of this transition include the appearance of intracellular membranes of the secretory pathway such as rough ER and Golgi complexes and a dramatic upregulation in expression and secretion of apolipoprotein E (ApoE; Tajima *et al.*, 1985; Auwerx *et al.*, 1988). To test the role of p180 in THP-1 cell differentiation, we utilized RNA interference (RNAi) to knockdown endogenous p180 levels to below half those of untreated cells.

The knockdown of p180 resulted in a severe decrease in the levels of intracellular membranes induced by phorbol ester treatment. The small numbers of membranes that were produced, both ER and Golgi, showed significant alterations in their fine structure, and in the case of the ER, ribosome binding. The most significant outcome was a 40–50% reduction in ApoE secretion that accompanied the RNAi-induced lowering of p180 expression in these cells.

Thus far, only the expression of p180, in yeast, resulted in the accumulation of rough ER membranes accompanied by increased levels of luminal and membrane markers, as well as downstream components of the secretory pathway (Wanker *et al.*, 1995; Becker *et al.*, 1999). On the basis of these observations, we hypothesized that the overexpression of p180 in a mammalian nonsecretory cell will induce the biogenesis of rough ER membranes as well as other components of the secretory pathway. To provide evidence that p180 is sufficient for the proliferation of rough ER membranes, we utilized a nonsecretory cell line, HEK293. Transient expression of p180 in HEK293 cells led to biogenesis of rough ER tubules and sheets and proliferation of Golgi complexes. Taken together, our results establish p180 as a key factor in stimulating the terminal differentiation of secretory cells.

MATERIALS AND METHODS

Cell Culture

THP-1 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 5×10^{-5} M 2-mercaptoethanol. THP-1 cells, 2.5×10^{-5} , were maintained in suspension in T75 flasks at 37°C in a

humidified environment with 5% CO₂. In all induction experiments, cells were treated to achieve a 350 nM (final concentration) of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). THP-1 cells were serum starved for 18–20 h, before TPA treatment. HEK293 cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. HEK293 cells were maintained in 10-cm Petri dishes, at 37°C in a humidified environment with 5% CO₂. HEK293 cells were transiently transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction manual. Transient expression of full-length p180 was achieved through the use of the mammalian vector pCDNA 3.1 that utilizes the hCMV promoter to drive gene expression (Invitrogen). For the construction of the full-length p180, we cloned BamHI-Sall fragments of the vector pRRFL (Wanker *et al.*, 1995) into pCDNA 3.1.

Cell Proliferation Assays

Numbers of THP-1 cells were monitored with the green fluorescent dye CyQuant (Invitrogen; Jones *et al.*, 2001). Because CyQuant stains both DNA and RNA, a more accurate cell count was obtained by pretreatment of all samples with DNase-free RNase. Quantitative differences in CyQuant fluorescence emission of untreated versus TPA-treated cells were measured using standard fluorescein excitation (485 nm) and emission (530 nm) wavelengths.

Fluorescence Microscopy

Detection of calnexin by indirect immunofluorescence was carried out according to Reggio *et al.* (1983). A Nikon ELIPS TE200 inverted microscope (Melville, NY) with a Nikon 100×/1.30 oil objective lens and fluorescent filters with 345-nm (DAPI), 489-nm (enhanced green fluorescent protein [eGFP]) and 589-nm (Texas Red) excitation wavelengths were used. Images were collected using SPOT advanced software (Diagnostic Instruments, Sterling Heights, MI). Untreated THP-1 cells were plated on 0.1% poly-L-lysine-coated glass coverslips and incubated overnight before processing. The extent of rough ER labeling around the nuclear envelope was estimated by counting test squares occupied by ER-specific fluorescence (calnexin labeling) on collected images.

Transmission Electron Microscopy

THP-1 cells were fixed with 2.5% glutaraldehyde buffered in 100 mM sodium cacodylate buffer (pH 7.4). Microwave-assisted processing and embedding of samples was conducted in accordance to Webster (2007).

Stereological Analysis

Volume densities (V_v) of rough ER and Golgi were quantified using published methods (Gundersen *et al.*, 1988). Briefly, test line lattices were superimposed over electron micrographs, and point counting was performed.

RNA Isolation and Northern Blotting

RNA isolation was performed using Trizol reagent. Total RNA (20 ng) was separated on a 1.2% formaldehyde-containing agarose gel and transferred to MagnaGraph nylon membranes (Micron Separations, Westborough, MA). Probes were generated from PCR using the following primer pairs, using cDNA isolated from THP-1 cells as a template: p180 forward, GCA AAC TGA GGG AGC TCA AC; p180 reverse, CTG TGT CTG CTC ATC CTC CA. Quantitative Northern blotting was performed using Image Quant software (Molecular Dynamics, Sunnyvale, CA).

Cell Fractionation and Western Detection

Both PMA-treated and untreated THP-1 cells were washed with PBS before homogenization in hypotonic buffer (10 mM HEPES, pH 7.5, 5 mM MgCl₂, and 1 mM ZnCl₂). After homogenization, sucrose was added to a final concentration of 0.25 M. Nuclei and cell debris were removed by centrifugation at $1000 \times g$ for 10 min. A crude membrane fraction was prepared from the postnuclear supernatant by centrifugation at $100,000 \times g$ for 1 h. The pellet was dissolved in 50 mM Tris HCl, pH 7.5, 250 mM KCl, 0.5% (wt/wt) Triton X-100, 0.2% gelatin, and a protease inhibitor cocktail. All Western blots were carried out on either 10% or 4–12% gradient Bis-Tris acrylamide gels. Monoclonal anti-calnexin (Calbiochem, La Jolla, CA) was used as the primary antibody, and ECL anti-mouse IgG coupled to horseradish peroxidase (HRP) was used as the secondary. Chemiluminescence was used for detection of labeled proteins.

Small-Hairpin RNA Lentiviral Vectors and THP-1 Cell Transduction

Lentiviral vectors (provided by Dr. Irvin S.Y. Chen, UCLA AIDS Institute) were used to introduce small-hairpin RNA (shRNA) for gene silencing of p180. The online tool "RNAi Oligoretriever," made available by G. J. Hannon's laboratory (Cold Spring Harbor Laboratory), was utilized to input a gene sequence and receive the hairpin-specific PCR primers as output. Two appropriate 27–29-nt-long p180 sequences were obtained and were spread throughout the p180 coding region that contain the appropriate flanking

bases to be useful for shRNA-based gene silencing. PCR was used (Castanotto *et al.*, 2002) to generate two sets of products (having the necessary 5' U6 universal primer, CCA AGG TCG GGC AGG AAG AGG GCC T; a sense strand, a stem loop, an anti-sense strand and the appropriate restriction sites) that were subcloned into lentiviral vectors for transduction into undifferentiated THP-1 cells. The two shRNA target sequences were as follows: shRNA 5 primer 1, CCA AGC TTC GGG AGA GTG AGG AGG CCC TGC AGA AGC GCG GTG TTT CGT CCT TTC; shRNA primer 2, TAT ATA CTG CAG AAA AAA ACA CTT CTA CAG AGC CTC CTC ACT CTC CCC AAG CTT; shRNA 6 primer 1, GCA AGC TTC CAG AGA ACT CCC AGC TCA CAG AGA GAA TCG GTG TTT CGT CCT TTC CAC AA; and shRNA 6 primer 2, TAT ATA CTG CAG AAA AAA GAT CCT CTC CGT GAG CTG GGA ATT CTC CGC AAG CTT CC. For the production of lentiviruses, 293T cells were cotransfected with 5 μ g of envelope plasmid, pHCMV-G; 12.5 μ g of packaging construct pCMVdeltaR8.2DVPR; 12.5 μ g of shRNA expression construct, pRRL-U6sLuc-cppt-PGKGF-SIN. The following day the medium was changed and subsequently left for an additional 36–48 h and finally filtered (0.4 μ m) in the presence of Polybrene (Millipore, Billerica, MA). Next, half the volume of filtered viral supernatant was added to $0.5\text{--}1.0 \times 10^6$ cells in six-well plates that were then centrifuged at 1800 rpm for 45 min at room temperature, followed by a 6-h incubation. This procedure was repeated twice. Transduced cells were analyzed and sorted for eGFP fluorescence by FACS after 8–10 d. It should be noted that transduced THP-1 cells expressing the highest levels of eGFP were sorted and further expanded in culture, for use in all experiments.

ApoE ELISA Assays

For the analysis of secreted ApoE, a specific ELISA was used. An anti-apolipoprotein E mAb (Chemicon International, Temecula, CA) was adsorbed (1:500) onto 96-well polystyrene plates and incubated at 4°C for 48 h. Supernatants from induced THP-1 cells or ApoE standard (recombinant Human apolipoprotein E, Academy Biomedical Company, Houston, TX) were added to each well and incubated at 37°C for 2 h. After the first incubation, unbound antigen was removed by extensive washing with TBS. An HRP-conjugated anti-ApoE polyclonal antibody (Academy Biomedical Company) was added (1:1000) and incubated at 37°C for 1 h. Unbound HRP-conjugated antibody was washed away with TBS, and a substrate solution containing *o*-phenylenediamine (OPD) was added to each well. The reaction was terminated by the addition of sulfuric acid, and the absorbance was measured at 490 nm, within 30 min. The concentration of ApoE in the sample was determined using a standard curve.

RESULTS

The human monocytic leukemia cell line (THP-1) undergoes a differentiation process leading to macrophage-like cells after stimulation by the phorbol ester TPA. Several hallmarks of the transition from monocytes to macrophages can be observed within 24 h after stimulation, including one round of cell division followed by exit from the cell cycle and entry into a quiescent state (Hass, 1992, 1993), a change in cell shape and adherence, the development of rough ER and Golgi, and the acquisition of an ability to phagocytose yeast or IgG-coated sheep erythrocytes (Tsuchiya *et al.*, 1982). Of relevance is the fact that stimulated THP-1 cells dramatically increase their ability to synthesize and secrete ApoE into the culture medium (Tajima *et al.*, 1985).

To first demonstrate that the attributes of THP-1 terminal differentiation were faithfully represented in our system, a series of cell biological, morphological, and biochemical analyses were performed. Figure 1A shows a growth curve indicating a doubling of cells in roughly 25 h, with only a minority of cells still dividing after this point. This is consistent with published results showing that 80% of THP-1 cells were able to respond to TPA treatment by arresting further growth and division and becoming adherent to the substratum (Tsuchiya *et al.*, 1982). A scanning electron microscopic (EM) comparison of control and treated cells showed uninduced THP-1 cells to be small, round, and poorly adherent (Figure 1B, left), whereas stimulated cells are flattened and adhere strongly to the substratum (Figure 1B, right). Corresponding transmission electron micrographs show uninduced cells to have typical lobated nuclei and a cytoplasm virtually devoid of intracellular organelles

(Figure 1C, left). By contrast, THP-1 cells stimulated for 24 h with TPA are larger and possess a well-developed rough ER composed of flattened, equally spaced cisternae (Figure 1C, center and right).

Stimulation of THP-1 cells led to the proliferation of rough ER (calnexin-positive) membranes extending from the perinuclear area to the cell periphery (Figure 1D). Samples of THP-1 cells were collected before TPA stimulation and at different time intervals after treatment (6, 12, 20, 48, and 72 h). These results, obtained using immunofluorescence, indicate that before TPA treatment ($T = 0$), rough ER is mainly represented by the nuclear envelope, consistent with our EM analysis (Figure 1C, left). Once stimulated by TPA, rough ER more typical of secretory cells appeared as early as 6 h after the start of treatment and continued to proliferate through the duration of the experiment. The appearance of binucleated THP-1 cells (indicative of mitosis) increased at around 20 h after TPA stimulation.

The morphological data on the expansion of the rough ER was corroborated by an increase in the levels of calnexin on a per cell basis, measured by Western blotting of whole cell lysates. As can be seen in Figure 1E, calnexin content increased over 72 h after TPA stimulation, reaching a plateau corresponding to a roughly fivefold increase compared with noninduced cells. These data suggested, but did not confirm that calnexin increases were due to additional calnexin being incorporated into existing membranes, as opposed to new membranes being proliferated with a normal complement of calnexin. The morphological data support the latter alternative. Increases in the concentration of calnexin in existing membranes predicts that an increase in calnexin levels would be observed if Western blots were performed on a per milligram protein basis when TPA stimulated cells were compared with controls. The alternative, the proliferation of new, potentially functional membranes, would show identical levels of rough ER-resident proteins between these two cell types on a per milligram protein basis. As can be seen in Figure 1, F and G, the levels of two rough ER markers, Sec61p (a component of the ER's translocon) and calnexin were unchanged when assayed on a per mg protein basis during TPA-induced membrane proliferation, supporting the notion that the increases in calnexin seen in Figure 1E were due to the biogenesis of new membranes. Of interest is the fact that p180 levels were dramatically increased, on a per milligram protein basis at 72 h of TPA treatment (Figure 1F), an occurrence whose significance will be addressed in the *Discussion*.

If p180 plays a central role in establishing a secretory phenotype it would be expected that its expression would increase in response to TPA stimulation as evidenced by the data presented in Figure 1F. Accordingly, both p180 mRNA and protein levels were assessed in the THP-1 cell system. Levels of p180 mRNA initially decrease, virtually disappearing at 12 h (Figure 2A). By 24 h, however, mRNA levels had rebounded, increasing to 200% of the initial value. Moreover, Western blotting of total membrane extracts, normalized to calnexin, demonstrated that p180 protein levels were also diminished at 12 h after TPA addition, but recovered quickly (24 h), reaching substantially higher levels by the 48 and 72 h time points (Figure 2B). Interestingly, p180 appears as a doublet in the blots, with the band of lower apparent molecular weight being prevalent during the period of p180 diminution. Previous *in vitro* studies have shown that p180 can exist as a functional 160-kDa proteolytic fragment (Savitz and Meyer 1990). It is possible that decreases in p180 mRNA and protein levels, as well as apparent molecular weight coincide with certain aspects

Figure 1. TPA treatment of THP-1 cells leads to their terminal differentiation into macrophage like cells. (A) Cell proliferation assay of THP-1 cells after TPA treatment. (B) Scanning electron micrographs of untreated (left) and TPA-treated THP-1 cells (right) at identical magnifications; bar, 5 μ m. (C) Transmission electron micrographs of untreated (left panel) and TPA-treated THP-1 cells (middle and right panels); bars = (a and B) 2 μ m; (C) 1 μ m. (D) A time-course experiment analyzing the rough ER-resident membrane protein, calnexin, during terminal differentiation of THP-1 cells after TPA treatment. Anti-calnexin antibody (red stain) was used to detect the rough ER at before ($t = 0$) and 6, 12, 20, 48, and 72 h after TPA stimulation. Nucleic acids were detected with DAPI (blue). (E) Western analysis detecting calnexin levels at 0, 6, 12, 24, 48, and 72 h after TPA treatment on a per cell basis. (F and G) Western blot of whole cell lysates, detecting p180 and Sec 61p (F) as well as calnexin (G), on a per milligram protein basis.

Figure 2. p180 expression during terminal differentiation of THP-1 cells. (A) Northern blot detecting p180 mRNA levels at 0, 2, 4, 12, and 24 h after TPA treatment. (B) Western blot detecting p180 protein levels in membrane fractions isolated from THP-1 cells at 0, 6, 12, 24, 48, and 72 h after TPA treatment. (C) ELISA quantification of secreted ApoE at 0, 24, 48, and 72 h after TPA treatment.

of cell division, as our data on THP-1 cells show that most of the ER is initially restricted to the nuclear envelope, which disassembles during mitosis. The possibility of decreases in p180 being synchronized with cell division will be addressed in the *Discussion*.

The gold standard of a transition to the secretory phenotype is the acquisition of the ability to secrete proteins at high levels. To demonstrate that the THP-1 cells in our experiments were capable of achieving this standard, their ability to secrete a major macrophage marker ApoE was examined. Measured by ELISA, ApoE in the culture medium increased steadily in the 72-h period after TPA addition (Figure 2C), roughly in parallel with the appearance of membranes and increases in levels of p180 (Figures 1C and 2B). With these hallmarks established, we proceeded to ex-

amine them in light of diminished intracellular levels of p180.

To directly demonstrate an involvement of p180 in the establishment of a secretory phenotype in THP-1 cells, RNAi technology was used to reduce intracellular p180 levels. A computer program was used to select potential sequences within the p180 coding region that would satisfy the requirements for making shRNA and lentiviral vectors were used to introduce the shRNA-generating sequences into the THP-1 cells (see *Materials and Methods*). Three populations of cells were utilized in this study. Controls included untransduced THP-1 cells as well as cells harboring a lentiviral vector containing an shRNA sequence (shRNA5) that was found to be ineffective in reducing levels of mRNA encoding p180 (Figure 3A). Cultures of the third type of cell showed marked reductions in p180-encoding mRNA through the introduction of a lentiviral vector encoding a sequence termed shRNA6, whose expression resulted in a reduction of p180 mRNA, compared with either control, within 8–10 d after transduction (Figure 3A). Densitometry analysis revealed that the reduction of 65–70% in specific mRNA levels by shRNA6 was observed in both TPA-treated and untreated cells. RNAi-mediated depletion of levels of p180 by 75% was confirmed by Western blotting (Figure 3B).

The reduction in p180 levels in THP-1 cells resulted in a number of morphological and biochemical changes relevant to the acquisition of a secretory phenotype. p180-deficient cells showed a decrease in levels of ER membranes, using calnexin as a marker (Figure 3C). Normalized to actin, and comparing TPA-stimulated cells, the level of reduction approximated 30–32% compared with controls. A change in measurable calnexin could result from either a loss of calnexin expression or a reduction in overall ER biogenesis that accompanies reduced p180 expression. To clarify, cells of the three types (both TPA-treated and untreated) were examined for rough ER proliferation in immunofluorescence studies using an anti-calnexin antibody as described above. A major reduction in calnexin-positive ER membranes was observed in TPA-treated cells with reduced p180 levels (Figure 3D). Morphometric analysis of a large sampling of cells was used to quantify the reduction in cell area occupied by rough ER in shRNA6 cells (Figure 3E). The results indicated a threefold drop in visible rough ER when p180 levels are diminished by shRNA6, compared with controls (THP-1 and shRNA5) in TPA-stimulated cells. Of interest was the consistently obtained and potentially relevant observation indicating that the space occupied by the nucleus in p180-deficient cells was considerably greater than controls, as indicated by DAPI staining (Figure 3D). The potential relevance of p180 in nuclear membrane integrity appears in the *Discussion*.

Significant alterations in rough ER abundance and morphology were observed in p180 deficient cells at the EM level. Typically, TPA-stimulated THP-1 cells possess extensive arrays of flattened rough cisternae typical of the ER-membranes of secretory cells (Figure 4, A and B). In contrast, shRNA-reduction of intracellular p180 levels resulted in three readily visible changes: 1) fewer membranes; 2) a more tubular/vesicular morphology; and 3) a virtual absence of membrane-bound ribosomes, i.e., new membranes resembled smooth ER (Figure 4, C and D). These features would be predicted to result through the loss of a protein that is involved in the maintenance of the integrity of the rough ER, as well as in its ability to bind ribosomes, two of the functions that we have shown and/or postulate to be functions of p180 (Savitz and Meyer 1990; Wanker *et al.*, 1995; Becker *et al.*, 1999).

Additionally, Golgi morphology was significantly altered in cells with lowered levels of p180 (Figure 4, E–H). TPA-

Figure 3. Introduction of shRNA suppresses p180 and ER expression levels in TPA-treated cells. (A) Northern blot detecting p180 mRNA levels in mock-transduced THP-1, shRNA5 (RNAi-transduced control) and shRNA6 cells at 0 and 72 h after TPA treatment. RNA extracted from Caco2 cells was used as a positive control for p180. (B) Western blot detecting p180 protein levels, from whole cell lysates, in mock-transduced THP-1, shRNA5 (RNAi-transduced control), and shRNA6 cells at 72 h after TPA treatment. (C) Western blot detecting calnexin protein levels, on a per cell basis, at 72 h after TPA treatment. Actin was used as a loading control. (D) Immunofluorescence microscopy of uninduced and 72 h TPA-treated THP-1, shRNA5 (RNAi-transduced control) and shRNA6 cells, respectively. An mAb specific for calnexin was detected with a secondary antibody conjugated to Texas Red. For nucleic acid staining DAPI was used (blue). Bars, 2 μm . (E) Mean area occupied by the ER (calnexin-positive membranes) in cells, estimated by point counting.

stimulated control cells (THP-1 and shRNA5) show extensive regions of Golgi membranes with many elongated, flattened cisternae and vesicles appearing to bud at the periphery of the cisternae (Figure 4, E and F). In the case of the stimulated shRNA6 cells, the Golgi regions have fewer membranes overall, and the cisternae are considerably shorter in length and more vesiculated (Figure 4, G and H). We performed stereological analysis to quantify the volume density (V_v) of the rough ER and Golgi (see *Materials and Methods*). It revealed that the V_v of rough ER and Golgi of shRNA6 cells is decreased by $\sim 50\%$ when levels of p180 are reduced, in comparison to control cells (Table 1). Taken together, these results provide additional evidence for the central role played by p180 in the biogenesis and maintenance of secretory membrane integrity during terminal differentiation.

To assess the impact of a p180 reduction on secretion itself, stimulated THP-1 cells of the three types were tested for their ability to accumulate ApoE in the medium. Based on an ELISA assay, total ApoE secretion was seen to drop by $\sim 40\%$ in cells with reduced p180, compared with controls (THP-1 and shRNA5), at 72 h after TPA induction (Figure 5A). This decrease in ApoE secretion was accompanied by an increase in the amount of ApoE that was retained intracellularly as measured by Western blotting (Figure 5, B and C). To further characterize the potential defect, cells were separated into membrane and cytosolic fractions, and ApoE was examined (see *Materials and Methods*). The cytosolic fraction from cells with lowered p180 revealed an accumulation of an intracellular unglycosylated form (Figure 5B, lane 3), whereas the membrane fraction contained both an unglycosylated and well as a glycosylated form (Figure 5C, lane 3). In contrast, control cells showed little to no cytosolic ApoE (Figure 5B, lanes 1 and 2), and their membrane fractions contained only minor amounts of the glycosylated form (Figure 5C, lanes 1 and 2). These data can be interpreted in the following manner. Loss of p180 reduces secretory capacity as evidenced by diminished secretion and accumulation of secretory product intracellularly. The reduction in functional ER in p180 deficiency leads to untranslocated and unglycosylated forms of ApoE accumulating in the cytosol and the reduction in functional Golgi results in glycosylated as well as unglycosylated forms appearing in membrane fractions, probably evidence of an inability to complete transport through the secretory pathway.

Previous studies have demonstrated that a number of human myeloid leukemia cells, including THP-1, progress through the cell cycle and replicate every 22–26 h. However, this progression is terminated after TPA treatment, resulting in the cell's exit from the cell cycle into a transient G0-arrest phase (Hass, 1992; Hass *et al.*, 1997). On entry into this quiescent state, within 48–72 h, THP-1 cells terminally differentiate and acquire a macrophage like phenotype (Hass *et al.*, 1993). One would predict, in the case of shRNA6 cells, that their loss in the ability to terminally differentiate a secretory phenotype would be accompanied by loss of entry in the quiescent state. Growth studies conducted after 72 h of TPA treatment, showed that p180-deficient cells continue to grow and divide in contrast to TPA-treated controls (THP-1 and shRNA5), behaving virtually identically to unstimulated cells (Figure 6). These data represent yet further evidence that numerous aspects of terminal secretory cell differentiation require normal levels of p180.

We were intrigued by the finding that the morphology of the rough ER in p180-deficient secretory cells showed a predominance of rough vesicles, as opposed to the rough cisternae found in controls. We hypothesized that p180 ex-

Figure 4, A–D. Lowered p180 expression leads to decreased rough ER and Golgi biogenesis. (A and B) Representative electron micrographs of 72 h TPA-treated THP-1 cells, showing elongated rough ER cisternae with bound ribosomes. (C and D) Representative electron micrographs of 72-h TPA-treated shRNA6 cells, showing vesiculated ER membranes virtually devoid of ribosomes.

pression levels dictate this aspect of rough ER morphology, with low levels favoring vesicles, high ones cisternae. It would follow then that increased expression of p180 in a nonsecretory cell system would lead to the biogenesis of rough vesicles and if sufficiently high, to rough cisternae. To see if p180 expression induces the biogenesis of rough vesicles and promotes the formation of cisternae, we transiently transfected a nonsecretory cell line (HEK293) cells with a mammalian expression vector encoding full-length p180 (see *Materials and Methods*). Electron micrographs of control untransfected HEK293 cells showed a cytoplasm virtually devoid of rough ER membranes and other downstream components of the secretory pathway, such as Golgi mem-

branes (Figure 7A). In contrast, electron micrographs of p180-transfected HEK293 cells revealed a significant increase in the biogenesis of rough ER-like vesicles and cisternae in the cytoplasm, reminiscent of highly active secretory cells such as pancreatic acinar or plasma cells (Figure 7, B and C).

The dramatic morphological change brought about in nonsecretory cells by p180 overexpression provides an insight into a potential role of p180 and the nature of the formation of rough ER cisternae. First, it would appear that rough vesicles or tubules are produced and align themselves opposite existing rough ER, in this case nuclear envelope (Figure 7C, arrows). This is followed by their fusion into a

Figure 4, E-H. (E and F) Representative electron micrographs of TPA-treated shRNA5 (RNAi-transduced control) cells, showing normal, elongated Golgi complexes. (G and H) Electron micrographs of TPA-treated shRNA6 cells, showing shortened and vesiculated Golgi complexes. Scale bars: (A-D and G) 500 nm; (E) 1 μ m; (F and H) 200 nm.

new cisternal or sheet-like, perinuclear layer of rough ER (Figure 7C, bracket). Then additional rough vesicles/tubules align themselves with the new layer, fuse and create another rough cisterna/sheet. This process would appear to repeat

itself for multiple rounds of cisterna/sheet formation as can be seen in the micrographs (Figure 7, B and C). Three-dimensionally, this would be analogous to adding layers to an onion. The spacing between the rough ER membrane

Table 1. Volume densities (V_v) of rough ER and Golgi in shRNA5 (RNAi-transduced control) and shRNA6 cells

	Total points rough ER	Total points section	V_v rough ER	Total points Golgi	Total points section	V_v Golgi
shRNA5	1,352	9,510	14.2%	546	4,022	13.6%
shRNA6	867	10,800	8.0%	264	3,684	7.2%

Figure 5. Reduction of p180 leads to decreased secretion of ApoE. (A) ELISA assay quantifying ApoE secreted into culture from control, shRNA5, and shRNA6 cells at 72 h after TPA treatment. (B) Western blot analysis detecting cellular ApoE levels in the cytosolic fraction. (C) Western blot analysis detecting cellular ApoE levels in membrane fractions.

cisternae/sheets appeared to be $\sim 104.3 \pm 7.5$ nm, typical of pancreatic acinar and plasma cells. This process would not occur or potentially even reverse itself in a cell whose p180 levels have been diminished. This is precisely what occurred in the experiments with THP-1 cells, where the equilibrium shifted to favor fewer rough cisternae/sheets and significantly increased levels of rough vesicles/tubules (Figure 4).

One can speculate that one function of p180, or possibly other ER proteins whose synthesis is stimulated by increased levels of p180, is to promote the homotypic fusion of

Figure 6. p180-deficient cells continue to proliferate after TPA treatment. Cell proliferation assay comparing shRNA5 and shRNA6 before and 72 h after TPA.

rough vesicles into rough cisternae. On the basis of the deleterious effects of p180 reduction on THP-1 cell structure and function, one would also predict that overexpression of p180 in a nonsecretory cell could promote increases in downstream elements of the secretory pathway such as the Golgi complex. Consistent with this prediction is the fact that electron micrographs of HEK293 cells transiently expressing p180 showed significant increases in Golgi biogenesis (Figure 7D). In contrast, control cells expressing empty vector showed no apparent increase in these membranes.

Studies of overexpression of p180 in yeast resulted in similar proliferation of rough ER cisternae and Golgi, as well as in the ability to secrete ectopically expressed proteins (Becker *et al.*, 1999). Although not addressed here, it is reasonable to predict that nonsecretory cells expressing sufficiently high levels of p180 would be able to increase their secretory capacity. The issue of increased secretion requires considerable additional effort and can be addressed in future studies.

DISCUSSION

In this study we have shown a direct link between the level of p180 and the ability of a presecretory cell to differentiate, as evidenced by changes in several hallmarks of this process. Reduced expression of p180 prevented cells from proliferating needed membranes, led to defects in processing and secretion of ApoE, and resulted in a failure of these cells to attach to a substratum and enter a quiescent state of cell growth. Additionally, transient expression of p180 in a mammalian nonsecretory cell line led to proliferation of rough ER membranes and Golgi complexes. From these data we conclude that without normal levels of p180, terminal differentiation of secretory cells is impossible.

The primary structure of p180 (Wanker *et al.*, 1995), as well as functional studies in yeast (Becker *et al.*, 1999), offers a basis for the interpretation of the effects of a p180 knock-down and its overexpression in mammalian cells. p180 is anchored in the ER membrane by a stretch of 28 hydrophobic and uncharged amino acid residues at its N-terminus, with the remainder of the protein having a cytosolic disposition (Savitz and Meyer 1990; Wanker *et al.*, 1995). Close to the N-terminus is a highly basic region containing a consensus decapeptide (NQGKKAEGAP) repeated 54 times without interruption, comprising its ribosome-binding domain. This region has also been shown to be necessary for p180's ability to stabilize mRNA that is targeted to the ER membrane (Hyde *et al.*, 2002). The C-terminal, highly acidic half of the protein is predicted to form numerous coiled-coils and is reminiscent of proteins that function as "tethers" and participate in homotypic membrane fusion events (Leung *et al.*, 1996; Diefenbach *et al.*, 2004; Ogawa-Goto *et al.*, 2007). Lastly, the C-terminal portion of p180 contains a kinectin-binding consensus sequence, suggesting a role for microtubule motors in some aspect of its function.

On the basis of these structural attributes, we propose the following scenario that accompanies up-regulation of p180, either naturally during THP-1 cell differentiation or induced by overexpression in nonsecretory cells. Increased expression and synthesis of p180 will first lead to increased levels of its incorporation into the membranes of the rough ER, via its N-terminal insertion sequence. Supported by data from yeast, an immediate effect of an increase in p180 content will be the stabilization (prolongation of half-life) of mRNA-encoding proteins that are targeted to the ER, both secretory and membrane (Hyde *et al.*, 2002), leading to their increased translation. Coincidentally, suggestions that mRNA turn-

Figure 7. Expression of p180 leads to rough ER and Golgi membrane biogenesis. (A) Representative electron micrograph of HEK293 cells transfected with vector control, showing the absence of rough ER and Golgi membranes. (B and C) Representative electron micrographs of HEK293 cells transiently transfected with p180 cDNA, showing elongated ribosome-studded ER cisternae extending from the nucleus to the cells periphery. Arrowheads indicate rough tubules aligned with the NE; bracket points out fused tubules having created rough ER sheets aligned with the NE. (D) Representative electron micrographs of HEK293 cells transiently transfected with p180 cDNA, showing the proliferation of Golgi membranes. Bars, 0.5 μm .

over rates decrease in mammalian cells because of ER targeting have already been reported (Tajima *et al.*, 1985; Wiest *et al.*, 1989; Basheeruddin *et al.*, 1992; Stephens *et al.*, 2005), consistent with such a role for p180. A major consequence of an increase in synthesis of ER-targeted proteins, whose expression levels have been increased due to p180-induced mRNA stabilization, would be a crowding of the rough ER membrane and lumen with proteins. To survive this dramatic increase in membrane protein, the cell must compensate by increasing the synthesis of lipids to enable an expansion of existing ER membrane and return to a healthy protein:lipid ratio. Confirmation of a requirement for increased lipid biosynthesis comes from published studies in yeast which demonstrated that p180-induced membrane biogenesis fails to occur in cells lacking the *INO2* gene, a master regulator of lipid biosynthesis (Block-Alper *et al.*, 2002). Virtually all of the lipid biosynthetic gene sequences that have been examined have *INO2* regulatory elements in their promoter sequences (Lopes and Henry 1991; Nikoloff *et al.*, 1992; Ambroziak and Henry, 1994; Nikoloff and Henry, 1994; Koipally *et al.*, 1996). This group includes a multitude of enzymes and transporters involved in lipid and sterol

metabolism (Greenberg and Lopes, 1996; Henry and Patton-Vogt, 1998). Once produced, these additional rough ER elements become integrated into the expansive cisternal ER network of secretory cells via homotypic fusions mediated by the C-terminal coiled-coil regions of p180. This paradigm, based on the multifunctionality of p180, explains how the expression of a single protein could have such profound and far-reaching effects on cellular morphology and function.

The striking micrographs of Figure 7, where p180 is over-expressed in a nonsecretory cell, show rough ER tubules (tubules) in close apposition to a vast array of newly synthesized rough ER sheets (sheets). Although part of a static image, it is clear that the tubules have aligned themselves with equal spacing to existing sheets, including the nuclear envelope (NE). Throughout the images there appear to be intermediate structures, i.e., nascent sheets that appear to have arisen from tubules in their proximity. The logical conclusion is that rough tubules are formed upon over-expression of p180. These tubules align themselves with defined spacing with existing sheets and fuse to form new sheets. The new sheets retain the same even spacing that was seen between tubules and the sheets with which they

had become aligned. In this way, layer upon layer of rough ER can be synthesized, ultimately leading to the “wall-to-wall” ER morphology reminiscent of highly active secretory tissues as evidenced by pancreatic acinar or plasma cells. Recent studies have documented that members of a specific protein family, the reticulons, are essential for the formation of tubules (Shibata *et al.*, 2008) and are lost in the transition to sheets (Shibata *et al.*, 2006). This would predict that the tubular morphology would require a significant increase in reticulon expression accompanying increased ER synthesis. Interestingly, microarray studies (Benyamini and Meyer, unpublished results), which were carried out on THP-1 cells that had been stimulated for 72 h with TPA, revealed roughly 50% increases in mRNA levels of reticulons 2 and 4 and a 97-fold jump in the transcript level of reticulon 1. It is also interesting to note that the tubules seen in these studies were uniformly covered with ribosomes, in contrast to those observed in yeast and/or in vitro (Voeltz *et al.*, 2006). This is more than likely due to the high-affinity ($K_d = 1.5 \times 10^{-8}$) ribosome-binding capacity of p180 (Savitz and Meyer, 1990).

p180, via its coiled-coil C-terminal domain, may also play a role in the process whereby ER membrane tubules fuse into evenly spaced sheets. Recent studies have shown that in the case of the flattened stacks of Golgi membranes, coiled-coil proteins of the golgin family are involved in the tethering of membranes and maintenance of the typical architecture of the Golgi complex (Short *et al.*, 2005).

Structural elements of p180 suggest yet another process in which p180 may play a role: the organization of the ER within the cytoplasm. A common method whereby cells accomplish the spatial organization of their organelles is through association with the cytoskeletal scaffolding. In animal cells the ER is intimately associated with microtubules. Several groups have shown that depolymerization of microtubules led to retraction of the ER from the cell periphery toward the NE (Georgatos *et al.*, 1997). This raises the question as to whether the kinectin-like, coiled-coil domain of p180 associates with the cell's cytoskeleton to localize rough vesicles/tubules and organize their assembly into rough ER membrane arrays, extending from the nucleus to the cell periphery. Supporting studies conducted by Ogawa-Goto *et al.* (2007) revealed that overexpression of p180 in mammalian cells enhanced microtubule acetylation and bundle formation. In vitro sedimentation assays showed that p180 directly bound to microtubules and possessed a microtubule-binding domain (Ogawa-Goto *et al.*, 2007).

Microtubule-associated cytoplasmic motor proteins such as kinesin and dynein mediate the movement of membranes along the cytoskeletal scaffolding (Sheetz, 1999). A number of studies have demonstrated that the motor protein kinesin tightly associates with microtubules and orchestrates the formation and breakdown of branching ER cisternae, as well as dismantling and reassembly of the nuclear envelope (Baumann and Walz, 2001). Through the use of a yeast two-hybrid system, Diefenbach *et al.* (2004) have demonstrated that the C-terminal domain of p180 and kinesin consist almost entirely of heptad repeats, corresponding to amino acid residues 1294–1413 in p180. The interacting regions of p180 and kinesin are homologous to the previously identified kinesin/kinectin-binding site, suggesting that p180 is important for trafficking of rough ER-like vesicles to other compartments within a cell. In conjunction with published reports, our results may suggest that rough ER-network formation is preceded by pulling of microtubule-bound membranes, presumably through a direct and/or indirect interaction between p180's C-terminal domain and kinesin (Diefenbach *et al.*, 2004; Ogawa-Goto *et al.*, 2007), followed

by their p180-mediated localization and fusion with pre-existing rough membranes to form rough ER cisternae/sheets.

Interestingly, characterization of mRNA granule transport intermediates in neurons revealed that they all contained kinesin KIF5 and were distinguished by the presence of bound ribosomes (Ohashi *et al.*, 2002; Mallardo *et al.*, 2003; Macchi *et al.*, 2003). These published results suggest that these mRNA granules containing bound ribosomes are most likely transported by a motor complex-dependent association between kinesin, KIF5, and the heptad repeats residing in the C-terminus of p180. However, further determination of the composition of mRNA-transported granules such as those found in neurons and mitotic cells is necessary to provide evidence linking p180 expression to rough vesicle localization.

Morphologically, the ER is a highly dynamic network of membrane tubules and flattened cisternae that is contiguous with the outer membrane of the nuclear envelope (Burke and Ellenberg, 2002; Voeltz *et al.*, 2002; Shibata *et al.*, 2006; Anderson and Hetzer, 2007). On the basis of these observations, one can predict that disruption of ER biogenesis would also lead to the loss of NE integrity. Our immunofluorescence studies, detecting the ER-resident protein calnexin, provide evidence that the nucleus of TPA-induced p180-deficient THP-1 cells is significantly enlarged compared with controls (Figure 3D). Preliminary studies using immunofluorescence, in which the nuclear envelope resident protein lamin-A was examined, revealed significant cytoplasmic staining for lamin-A in p180 knockdown cells, suggesting NE breakdown. In contrast, anti-lamin-A staining in control cells showed strong signals around the nucleus with minimal to no staining in the cytoplasm (Benyamini and Meyer, unpublished results).

How is p180 involved in maintaining the structural integrity of the nuclear envelope? We have postulated that the C-terminal coiled coil domains of p180 mediate homotypic membrane fusion of rough ER vesicles/tubules to form larger cisternae/sheets and by extension help to reform the nuclear envelope after mitosis. A dynamic equilibrium between these states of vesicles/tubules or cisternae/sheets is regulated by the presence of p180; higher p180 levels are required to promote cisterna/sheet (or nuclear envelope) formation, whereas reduction in p180 levels favors vesicle/tubule formation from cisternae/sheets as well as disassembly of the nuclear envelope. This hypothesis is supported by our data. There is a naturally occurring decrease in p180 levels during the cell cycle immediately before cell division, which is also supported by distended nuclei in the absence of p180 (Figures 1D and 3D). Moreover, we observed that when p180 levels are reduced, the vesicular—as opposed to the cisternal—state is favored (Figure 4).

Taken together, the data presented here provide strong evidence for many roles for p180 in the coordination of the growth and terminal differentiation of secretory cells. In the normal course of differentiation, levels of p180 are initially diminished and then rise significantly as the cell enters a quiescent growth state and exhibits extensive membrane proliferation and dramatic increases in secretory activity. The loss of p180 compromises all of these attributes leading to a loss of entry into quiescence, an inability to synthesize morphologically correct ER and Golgi membranes, a loss of membrane-bound ribosomes, and most importantly a substantial decrease in secretory activity. Consistent with these observations and our hypothesis was the fact that the overexpression of p180 resulted in a significant proliferation of rough ER and Golgi membranes, two hallmarks of establishing a secretory phenotype.

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