Nongenomic STAT5-dependent effects on Golgi apparatus and endoplasmic reticulum structure and function

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Lee JE, Yang YM, Liang FX, Gough DJ, Levy DE, Sehgal PB. Nongenomic STAT5-dependent effects on Golgi apparatus and endoplasmic reticulum structure and function. Am J Physiol Cell Physiol 302: C804–C820, 2012. First published December 7, 2011; doi:10.1152/ajpcell.00379.2011.—We report unexpected nongenomic functions of signal transducer and activator of transcription (STAT) 5 species in the cytoplasm aimed at preserving the structure and function of the Golgi apparatus and rough endoplasmic reticulum (ER) in vascular cells. Immunoimaging and green fluorescent protein-tagged-STAT5a protein localization studies showed the constitutive association of nonphosphorylated STAT5a, and to a lesser extent STAT5b, with the Golgi apparatus and of STAT5a with centrosomes in human pulmonary arterial endothelial and smooth muscle cells. Acute knockdown of STAT5a/b species using small interfering RNAs (siRNAs), including in the presence of an mRNA synthesis inhibitor (5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole), produced a dramatic phenotype within 1 day, consisting of dilatation and fragmentation of Golgi cisternae, a marked tubule-to-cyst change in the ER, increased accumulation of reticulin-4 (RTN4)/Nogo-B and atlastin-3 (ATL3) at cyst-zone boundaries, cystic separation of the outer and inner nuclear membranes, accompanied by scalloped/lunate distortion of the nucleus, with accumulation of RTN4 on convex sides of distorted nuclei. These cells showed inhibition of vesicular stomatitis virus G protein glycoprotein trafficking, mitochondrial fragmentation, and reduced mitochondrial function. STAT5a/b−/− mouse embryo fibroblasts also showed altered ER/Golgi dynamics. RTN4 knockdown using siRNA did not affect development of the cystic phenotype; ATL3 siRNA led to effacement of cyst-zone boundaries. In magnetic-bead cross-immunopanning assays, ATL3 bound both STAT5a and STAT5b. Remarkably, this novel cystic ER/lunate nucleus phenotype was characteristic of vascular cells in arterial lesions of idiopathic pulmonary hypertension, an unrelentingly fatal human disease. These data provide evidence of a STAT-family protein regulating the structure of a cytoplasmic organelle and implicate this mechanism in the pathogenesis of a human disease.

human pulmonary arterial endothelial and smooth muscle cells; small interfering RNA knockdown; enlargement and fragmentation of Golgi apparatus; cystic dilatation of endoplasmic reticulum; intracellular trafficking

A FAMILY OF SEVEN MAMMALIAN proteins, dubbed “signal transducers and activators of transcription” (STAT1, 2, 3, 4, 5a, 5b, and 6) was identified in the 1990s by their ability to mediate cytokine and growth factor-induced transcriptional activation of target genes in the cell nucleus (23). These proteins have a predominant cytoplasmic provenance, but constitutively shuttle, with or without Tyr and/or Ser phosphorylation, between the cytoplasm and the nucleus (21, 23). Phosphorylated STAT protein species produced in response to cytokine and growth factor stimulation have a greater affinity for DNA elements in promoters of target genes, leading usually to increased gene transcription (21, 23). Nonphosphorylated STAT species, which constitutively shuttle to the nucleus, regulate transcription from a different set of target genes (21, 23). Thus, over the years, the nuclear transcriptional regulatory function of STAT proteins has commanded almost exclusive attention. To date, none of the STAT proteins has been implicated in maintaining the structural integrity of cytoplasmic organelles, transcriptionally or otherwise. However, there is growing consideration of “nongenomic” functions of STAT proteins in the cytoplasm, stemming from reports that cytoplasmic non-Tyr-phosphorylated STAT3 regulated mitochondrial respiratory function (6, 37). However, studies using fluorescently tagged STAT3 have failed to provide evidence for mitochondrial association (39). Herein we report the novel discovery that nonphosphorylated STAT5 species, especially STAT5a, appear to constitutively associate with the Golgi apparatus in mammalian cells in both immunofluorescence and green fluorescent protein (GFP)-tagged protein localization studies and explore the functional implications of this association using a small interfering RNA (siRNA)-mediated acute knockdown approach.

Of the STAT proteins, STAT5a was first identified by its ability to mediate prolactin-induced upregulation of the expression of milk proteins (5). Subsequently, STAT5b, a homolog 96% related to STAT5a, was isolated (17), and both STAT5a/b were shown to play essential redundant and nonredundant roles in mammary gland development, cytokine signaling, and immunoregulation in different tissues, but always in terms of regulating gene expression in the nucleus (7, 14). STAT5a/b−/− mice displayed impaired mammary gland development and the absence of lactation (7, 16, 18). STAT5b−/− mice displayed reduction but not absence of lactation and a sexual dimorphism in terms of body growth and liver gene expression (7, 35). STAT5a/b−/− double knockout mice had significant perinatal mortality, and those that survived had reduced body weight and were infertile (3, 7). The occurrence of “compensating” cytokine and growth factor signaling over time through other STAT transcription factors has been observed in tissues of respective STAT5a/b knockout mice (6, 16).

In initial immunofluorescence studies, we made the unexpected observation that STAT5a was constitutively associated with the Golgi apparatus in primary human pulmonary arterial endothelial (HPAEC) and smooth muscle cells (HPASMC).
This observation led us to consider nongenomic functions of STAT5 in cytoplasmic organelles, especially at the level of the intracellular membrane trafficking/secretory pathway. We were mindful of the newly emergent paradigm that interconnected the regulation of the structures and functions of the Golgi apparatus, endoplasmic reticulum (ER), and mitochondria to each other (4, 15). Moreover, in light of our laboratory’s previous demonstration of Golgi dysfunction in vascular cells in lung arterial lesions in idiopathic pulmonary arterial hypertension (IPAH) (22, 25), we investigated the implications of our observations in this human disease.

MATERIALS AND METHODS

Cell culture. Primary HPAEC and HPASMC cells were purchased from Clonetics (San Diego, CA). Both were seeded into T-25, T-75, or six-well plates coated with fibronectin, collagen, and bovine serum albumin (respectively, 1, 30, and 10 μg/ml in coating medium) (11).

HPAECs were grown in Medium 200 supplemented with low serum growth supplement (Cascade Biologics, Carlsbad, CA) and were used between passages 4 and 10. HPASMCs were grown in Medium 231 containing smooth muscle growth supplement (Cascade Biologics). For differentiation, HPASMC cultures were switched to Medium 231 supplemented with smooth muscle differentiation supplement (Cascade Biologics). HPAECs were verified using immunofluorescence assays to be positive for expression of von Willebrand factor and negative for smooth muscle α-actin; conversely, HPASMCs were verified to be von Willebrand factor negative and smooth muscle α-actin positive. Primary bovine pulmonary arterial endothelial cells were used as previously described (11, 12). EA.hy926 cells, derived from primary human umbilical vein endothelial cells immortalized by fusion with A549 cells, were obtained from Dr. Michal Schwartzman (Dept. of Pharmacology, New York Medical College) and were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% vol/vol fetal bovine serum and 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (11). Early passage STAT5a/b−/−

Fig. 1. Association of signal transducer and activator of transcription 5a (STAT5a) with the Golgi apparatus and centrosomes and STAT5b with the Golgi apparatus. A: cultures of primary human pulmonary arterial endothelial (HPAEC) and smooth muscle cells (HPASMC) were washed with a digitonin (50 μg/ml)/0.3 M sucrose buffer, fixed, and immunostained for STAT5a and the Golgi marker Golgi matrix 130-kDa protein (GM130). Insets show a higher magnification view of the Golgi region; white arrows point to centrosomes. Scale bars = 5 μm (as are in all figures, except where otherwise indicated). B: two-dimensional (2D) compilations of z-stack images (×100 oil objective) of the Golgi region in cells in the experiment in A; arrows point to centrosomes. C: HPAECs and HPASMCs were immunoprobred for STAT5b in association with GM130 (arrows). Insets show a higher magnification view of the Golgi region. DAPI, 4,6-diamidino-2-phenylindole.

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mouse embryonic fibroblasts (MEFs) were a gift of Dr. Lothar Henningerhausen (14), and these cells, as well as early passage wild-type (wt) MEFs, were grown in coated culture vessels in DMEM supplemented with 15% fetal bovine serum, 0.1 mM β-mercaptoethanol, and an additional ×1 supplement of nonessential amino acids. Phase-contrast microscopy of live cultures at the conclusion of each experiment was carried out using a Nikon Diaphot Microscope and a Nikon Coolpix digital camera.

The murine hybridoma clone producing the anti-VSV-G MAb (vesicular stomatitis virus G protein monoclonal antibody; clone II, stock 8G5F11) (13) was a gift from Dr. Douglas S. Lyles.

Plasmids and siRNAs. The full-length wt human STAT5a-GFP construct was purchased from Origene Technologies (Rockville, MD), and site-directed mutagenesis was carried out to make the mutants Y694A, S726A, and the double mutant Y694A/S726A. The mutants were verified by nucleotide sequencing and by characterizing the reactivity of the expressed proteins to respective anti-STAT5 antibodies. Plasmid transfections were carried out using PolyFect (Qiagen, Valencia, CA) and the manufacturer’s protocols. The tsO45-VSV-G-GFP expression vector (8) was a gift from Dr. Jennifer Lippincott-Schwartz. Respective siRNA oligonucleotides were purchased from Santa Cruz (see specific catalog numbers below). Acute knockdown of target proteins was carried out using siRNA transfection reagent (1:1 ratio of siRNA to transfection reagent). Typically 2–6 μl of a 10 μM siRNA stock and 2–6 μl siRNA transfection reagent were used per 35-mm plate or per well in a six-well plate. STAT5a/b double knockdown experiments were carried out using 2 μl of STAT5a siRNA and 2 μl of STAT5b siRNA, together with 4 μl of siRNA transfection reagent. Corresponding controls received 4 μl of scrambled siRNA mixed with 4 μl transfection reagent. Under these conditions, the scrambled siRNA transfections had no apparent effects on cells. Primary HPAECs were readily transfectable with siRNAs for STAT5a/b with 60–80% or more of cells in individual experiments showing phenotypic knockdown by immunofluorescence. In contrast, primary HPASMCs were difficult to transfect (1–5% transfected cells). Thus most of the siRNA experiments in this study were carried out using primary HPAECs.

Immunofluorescence and GFP-fluorescence microscopy of cells in culture. Cells in culture were fixed using cold paraformaldehyde (4%), without or after first washing the cells using a buffer containing digitonin (50 μg/ml)sucrose (0.3 M), as indicated in respective figure legends. Immunofluorescence assays were carried out using antibodies from specific sources and corresponding to specific catalog numbers, as enumerated below (dilution range 1:100 to 1:1,000) and as described earlier (11, 12). The cells were imaged as previously reported (11) using a Zeiss Axiosmager M2 motorized microscopy system with Zeiss WN-Achroplan ×40/numerical aperture 0.75 or Zeiss EC Plan-Neofluor ×100/numerical aperture 1.3 oil objectives equipped with an high-resolution RGB HRc AxioCam camera and AxioVision 4.8.1 software in a 1,388 × 1,040 pixel high-speed color-capture mode. Imaging using the ×100 oil objective included data collection using z-stack data acquisition software (typically stacks of 15 or 21 slices, 0.3 μm apart) and rendering the data using Inside4D modules in the Zeiss software. Controls included secondary antibodies alone, peptide competition assays, and multiple different antibodies toward the same antigen. All data within each experiment were collected at identical imaging settings.

Electron microscopy. Thin-section electron microscopy (EM) was carried out by the OCS Microscopy Core at NYU-Langone Medical Center in one of two ways: 1) HPAECs grown in 60-mm plates, the culture medium drained, then 1 ml of 1× fixative [paraformaldehyde (2%)]-glutaraldehyde (2.5%) in 0.1 M sodium-cacodylate buffer (pH 7.4) was added for 2 min at room temperature, the cells then scraped, pelleted, and further fixed for 1–2 h in 1× fixative at 4°C; or 2) 1 ml of 2× fixative was added to HPAEC cultures in 35-mm plates containing 1 ml culture medium, the cells incubated at 37°C for 2 min, the cultures drained, replenished with 2 ml of fresh × fixative and incubated at 37°C for 1 h, and then embedded in situ and sectioned en face.

Cell fractionation, magnetic-bead immunopanning, and Western blotting. Cell fractionation and gradient flotation methods used to prepare respective whole cell extracts, cytoplasmic extracts, as well as Golgi-enriched membrane fractions from endothelial cells in culture have been described previously (12, 24, 40, 12). Methods for magnetic bead immunopanning using Protein A-Dynal beads (Invitrogen, Carlsbad, CA) and Western blotting were as previously reported (Refs. 12, 24, 40, and citations therein).

Human lung sections. Paraffin-block sections (5 μm thick) of archived formalin-fixed human lung tissue from four IPAH patients and three control subjects used in the present study are from our laboratory’s previous studies (11, 25), were a gift of Dr. Rubin M. Tuder, and were provided without any individual identifiable markers, according to protocols reviewed and approved by the Institutional Review Board of the New York University School of Medicine.

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Review Board, Johns Hopkins University School of Medicine (Baltimore, MD). These sections carry the same letter designations as in the previous studies. The sections were processed for hematoxylino
eosin and immunolabelling, as reported previously (11, 25), and imaged using the Zeiss Axiomager M2 microscopy system, the ×100 oil objective, and the HRc AxioCam camera in a 1,388 × 1,040 pixel high-speed color-capture mode, including using z-stack data acquisition software (typically stacks of 15 or 21 slices 0.3 μm apart).

Quantitative image analyses. This was carried out using the McMaster Biophotonics Facility version of NIH Image J software and respective utility plugins (available as free downloads from www.
macbiophotonics.ca/imagej/) as reported previously (11). Statistical testing was carried out using the Student’s t-test and multiple-group ANOVA.

Chemicals, antibodies, and siRNA reagents (respectively catalog numbers in parentheses). The mRNA synthesis inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was purchased from Sigma-Aldrich (St. Louis, MO; D91916) and used at 60–70 μM (26). ER-Tracker (used at 1 μM for 30 min at 37°C) and tetrathia
damine ethyl ester (TMRE) (used at 5 nM for 15 min at 37°C) were purchased from Invitrogen (Carlsbad, CA; E-34250 and T-669, respectively). Rabbit polyclonal antibodies (PAbs) to giantin (24586) and atlastin-3 (ATL3) (104262) were purchased from Abcam (Cambridge, MA). Rabbit PAs to STAT5a (sc-1081x), STAT5b (sc-835x), PS-STAT5a/b (Ser726) (sc-12893-R), PY-STAT5a/b (Tyr694) (sc-101806), STAT3 (sc-482), and ATL1 (sc-67232); goat PAs to ATL2 (sc-109213), reticulon-4 (RTN4)/Nogo-B (sc-11027), and murine MAb to F1-ATPase (sc-58618), as well as the STAT5a peptide (sc-1081 P), caveolin-1 peptide (sc-894 P), and STAT3 peptide (sc-482 P) used in competition experiments were from Santa Cruz Biotechnology (Santa Cruz, CA). Murine MAbs to GM130 (Golgi matrix 130-kDa protein; 610823) was purchased from BD Biosciences (Eugene, OR). Respective AlexaFluor 488- and AlexaFluor 594-tagged secondary donkey antibodies to rabbit (A-11008 and A-11012), mouse (A-21202 and A-21203), or goat (A-11055 and A-11058) IgG were from Invitrogen Molecular Probes (Eugene, OR).

siRNA preparations and transfection reagents were obtained from Santa Cruz Biotechnology. These were siRNA transfection reagent (sc-29528), siRNA transfection medium (sc-36868), scrambled control-A siRNA (sc-37007), and siRNA preparations to STAT5a [human (h)] (sc-37008), STAT5b (h) (sc-37010), STAT5a [mouse (m)] (sc-37009), STAT5b (m) (sc-37011), STAT3 (h) (sc-29493), STAT6 (h) (sc-270188), nitric oxide synthase 3 (NOS3) (h) (sc-36903), α subtype of soluble NSF acceptor protein, where NSF is the N-ethylmaleimide-sensitive factor (α-SNAP) (h) (sc-29617), bone morphogenetic protein receptor type II (BMPRII) (h) (sc-40220), RTN4 (h) (sc-43974), and ATL3 (h) (sc-96376).

RESULTS

Constitutive association of nonphosphorylated STAT5 species with the Golgi apparatus. Immunofluorescense studies in primary HPAEC and HPASMC cultures that had been washed before fixation with a digitonin-sucrose buffer to remove bulk soluble STAT proteins revealed the constitutive association of STAT5a with the Golgi apparatus and centrosomes (Fig. 1, A and B). The Golgi-specific immunostaining was observed using both a PAb, as well as a different MAb, to STAT5a (and thus with two different secondary antibodies and two different Golgi markers: GM130 MAb and giantin PAb), was not observed using other irrelevant rabbit PAs as the primary, was competed away by specific STAT5a peptide, but not by irrelevant caveolin-1 peptide, and was diminished in cells transfected with STAT5a siRNA, but not scrambled siRNA (data not shown). The centrosome specificity of STAT5a immunostaining was confirmed by double immunostaining using MAbs

![Fig. 3. Apparent association of wild-type (wt) and phosphorylation-site mutants of human STAT5a-GFP (green fluorescent protein) with the Golgi apparatus in vascular cells of heterologous and homologous species. A: bovine pulmonary arterial endothelial cells (BPAECs) were transfected with an expression construct for wt human STAT5a-GFP, fixed 1 day later, and imaged in green, together with immunostaining for the Golgi tether giantin in red. Insets show the Golgi region at higher magnification. B: HPAEC and HPASMC cultures were transfected with the wt human STAT5a-GFP expression vector, fixed 1 day later, and imaged in green, together with giantin immunostaining in red. Arrows indicate the Golgi apparatus. C: respective wt and mutant STAT5a-GFP expression constructs were transfected into BPAECs, the cells fixed 1 day later, and the Golgi association of the GFP-tagged proteins (green) characterized by co-immunostaining for giantin (red). Arrows and insets highlight the Golgi apparatus.](http://ajpcell.physiology.org/).
for γ-tubulin (GTU88) and pericentrin (data not shown). STAT5b was also associated with the Golgi in both HPAECs and HPASMCs, but to a lesser extent, and not at all with centrosomes (Fig. 1C). Furthermore, in immunofluorescence studies, Golgi-associated STAT5a was not phosphorylated either at Tyr-694 or at Ser-726, but STAT5a that associated with the centrosome appeared as Ser-726-phosphorylated (Fig. 2). Additionally, human Hep3B hepatocytes and human EA.hy926 endothelial cells also showed STAT5a association with the Golgi apparatus and with centrosomes (data not shown).

Exogenously expressed GFP-tagged human STAT5a appeared to be associated with the Golgi apparatus in heterologous bovine endothelial cells (Fig. 3A), murine STAT5a/b−/− embryo fibroblasts (data not shown), and in homologous human cells (Fig. 3B). Moreover, phosphorylation-site point mutants of STAT5a-GFP (Y694A, S726A, and double-mutant Y694A/S726A) all appeared to associate with the Golgi apparatus (Fig. 3C). Additionally, STAT5a and STAT5b proteins were observed by Western blotting in subcellular fractions enriched in Golgi membranes (Fig. 4A) (22, 38). Taken together, these data using immunoimaging, localization of GFP-tagged-STAT5a, and subcellular fractionation suggested the association of STAT5a with the Golgi apparatus. This association appeared specific for STAT5 species in that none of the other five STAT proteins evaluated (STAT1, 2, 3, 4, and 6) were so observed (data not shown).

The cystic ER/dilated-fragmented Golgi phenotype upon acute knockdown of STAT5a/b. Acute siRNA-mediated knockdowns of STAT5a, or STAT5b, or both in HPAECs or HPASMCs produced a characteristic cystic phenotype evident within 1 day, even in the presence of the mRNA synthesis inhibitor DRB (24) (Figs. 5–9) with the earliest changes apparent by 8 h (data not shown). One day after siRNA transfection, by phase-contrast microscopy, the treated HPAECs displayed a lattice of cysts in the cytoplasm and lunate or scalloped distortion of nuclei with, typically, large cysts on one or both sides of the distorted nucleus (Fig. 5A). This phenotype was observed using the nominal STAT5a siRNA alone and the nominal STAT5b siRNA alone, but was observed in the highest number of cells in a culture when both siRNAs were used together. The cystic phenotype was not observed using scrambled siRNA (Figs. 5–8) or siRNAs directed against STAT3, STAT6, endothelial NOS, BMPRII, or α-SNAP (data not shown). Moreover, this phenotype was also observed in HPASMCs and Hep3B hepatocytes (data not shown), as well as EA.hy926 human endothelial cells (seen below in Fig. 13A). Murine-specific, but not human-specific, STAT5a/b siRNAs elicited the cystic phenotype in wt murine fibroblasts (data not shown). Western blotting confirmed the reduction of the respective STAT5a/b using the respective siRNA oligonucleotides, with an 80–90% loss by day 2 with some cross-reduction (Fig. 4, B and C). The cystic-cell phenotype was fully developed within 1 day i.e., at a time when there was ~70–85% reduction in STAT5a/b (Fig. 4B).

Three dimensional z-stack immunoimaging showed fragmentation of the Golgi apparatus after STAT5a knockdown in HPAECs with residual STAT5a still present in some of the Golgi elements (Figs. 5B and 6A). Similar Golgi fragmentation

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Fig. 4. Western blot data showing association of STAT5a and STAT5b with Golgi-enriched membrane fractions, the efficiency of STAT5a/b knockdown by respective small interfering RNAs (siRNAs), and the association of STAT5a and STAT5b with atlastin-3 (ATL3). A: STAT5a and STAT5b associate with a subcellular fraction enriched in Golgi membranes isolated using the flotation gradient method of Xu and Shields (40), as used in Sehgal et al. (24). Western blots of respective STAT5a and b proteins and the Golgi marker GM130 from the “Golgi” fraction derived from the 0.8/1.2 M sucrose interface from duplicate gradients run side by side are illustrated. B and C: the content of respective STAT5a and STAT5b proteins in cell extracts prepared from HPAECs either 1 day (A) or 2 days (B) after transfection with the indicated siRNAs. Equal amounts of total cellular protein (~14 μg/lane) were loaded in each of the four lanes in each gel; reprobing for β-actin confirmed equivalent protein loading in B. The densitometric quantitations (as % control) are of blots immediately below each graph. D: protein-A magnetic-bead cross-immunopanning (IPN) of STAT5a and STAT5b by anti-ATL3 polyclonal antibody (PAb) from extracts of EA.hy926 endothelial cells. IPN was carried out as indicated using anti-ATL3 rabbit PAb with controls, which included absence of any IgG (“beads”) and a negative PAb (anti-ATL1 PAb) (there is little if any ATL1 expressed in endothelial cells). Cell extract volumes used: leftmost lanes: 10 μl; for IPN: 1,000 μl.
was also observed in HPASMCs and after STAT5b knockdown alone and after STAT5a/b double knockdown, but not after STAT3 or STAT6 siRNA knockdowns (data not shown). Cytoplasmic dispersal of giantin, presumptively to the ER, was more extensive than that of GM130; additionally, different Golgi-region fragments showed both coincidence and divergence in giantin and GM130 presence (data not shown). Thin-section EM confirmed extensive Golgi dilatation and fragmentation after STAT5a/b knockdown (Fig. 5C). Immunostaining for two ER-resident proteins, the ER structural protein RTN4/Nogo-B and the ER-resident dynamin-family GTPase ATL3, showed dramatic changes in the ER in cells exposed to STAT5a/b siRNA (Fig. 6B). RTN4 accumulated in the intercystic ER membranes and particularly along the convex sides of the distorted nuclei (Figs. 6B and 7B). ATL3, the predominant isoform of the ATL family of ER-resident GTPases in HPAECs (17), accumulated markedly at cyst-zone boundaries (Fig. 6B).

Figure 7A confirms the changes in ER using live-cell ER-Tracker labeling. In these assays, the intercystic membranes stained with ER-Tracker (Fig. 7A). Figure 7, B and C, shows that the demarcation of the boundaries of the cystic ER change is associated with punctate accumulation of RTN4 at the tubule-to-cyst boundary. Moreover, Fig. 7B emphasizes the lack of effect of DRB, an mRNA synthesis inhibitor (26), on development of this phenotype. Thus the
events leading to the cystic phenotype do not involve transcription.

EM data provided definitive evidence that the observed cysts represented dilated rough ER elements studded on the cytosolic side with ribosomes (Fig. 7D, center panels), and that the large juxtanuclear cysts represented the peeling away and separation of the outer nuclear membrane from the inner nuclear membrane with disruption of nuclear pores (Fig. 7D, right panels, and Fig. 8).

Presumptive stages in the process by which ER tubules appear to transition into cysts are depicted in Figs. 7C and 9. Figure 7C illustrates the sharp demarcation of the tubule-to-cyst change at the cell periphery displayed by immunostaining for RTN4. Figure 9A shows the increased punctate deposition of RTN4 along ER tubules and the periphery of the zones of cystic change. The cystic change can commence in a location away from the cell center (Fig. 9A, bottom row) and can include a transition of the ER from tubule to cyst (Fig. 7C) or tubule to sheet and then to cyst (Fig. 9A, bottom right). A presumptive early dilatation of ER change observed by EM of in situ embedded HPAECs sectioned en face is illustrated in Fig. 9B, left, whereas a tubule-to-cyst transition region is shown in Fig. 9B, right.

Additionally, the STAT5a/b-siRNA-induced cystic ER zones showed evidence of mitochondrial fragmentation (Fig. 10). However, both by immunofluorescence imaging and by EM, there was little effect on centrosome structure at a time when the cystic ER/dilated Golgi alterations were already evident (data not shown).

ER/Golgi in STAT5a/b<sup>-/-</sup> MEFs. We then investigated the structure of the Golgi apparatus and ER in early passage MEFs derived from STAT5a/b<sup>-/-</sup> embryos (3, 7) compared with wt early passage MEFs. Survival and growth of early passage STAT5a/b<sup>-/-</sup> MEFs required an enriched medium [DMEM supplemented with 15% (vol/vol) fetal bovine serum, additional nonessential amino acids, and β-mercaptoethanol (0.1 mM)] compared with wt early passage MEFs, which grow very well in DMEM supplemented with 5% calf serum. At 3 h after fresh plating (range 1–6 h), even in enriched medium STAT5a/b<sup>-/-</sup> MEFs displayed a marked accumulation of perinuclear cysts (Fig. 11A) compared with wt MEFs. In live-cell staining, these cysts were ER-Tracker positive (Fig. 11B). One to two days later, the markedly cystic ER phenotype had subsided, but the STAT5a/b<sup>-/-</sup> MEFs had a larger cell size ("megalocytosis") with the persistence of an enlarged and more fragmented Golgi apparatus (Fig. 12). Subsequent transfection with mu-
rine-specific STAT5a/b siRNAs 2 days after replating (at a time when most of the cystic ER changes had resolved) did not elicit a cystic ER/lunate nucleus phenotype in STAT5a/b−/− MEFs, but did so in wt MEFs (data not shown).

Functional changes in STAT5a/b-knockdown cells. The functional consequences of Golgi fragmentation/cystic ER change on intracellular trafficking were investigated using the tsO45 temperature-sensitive VSV-G-GFP protein and a MAb
specific for a cell-surface-specific epitope in VSV-G (8, 13). Because this assay required cells to be kept at 39.5°C overnight following transfection with the VSV-G-GFP expression vector (this protein remains in the ER at this temperature), these experiments were carried out in the hardier EA.hy926 endothelial cells, which survive the higher temperature. Figure 13, A–C, shows that, upon shiftdown to 32°C for 1 h (which now allows trafficking from ER to Golgi to plasma membrane), the VSV-G-GFP in almost all control cells transited to the cell surface. However, VSV-G-GFP in cells showing a cystic change on STAT5a/b siRNA transfection showed markedly reduced transit to the plasma membrane (Fig. 13, A–C). In some cells with a cystic change, the VSV-G-GFP remained in the ER (Fig. 13A, middle row), while, in others, VSV-G-GFP transited to the Golgi fragments, but did not reach the cell surface (Fig. 13A, bottom row). It is noteworthy from the data in Fig. 13C that cells transfected with STAT5a/b siRNA, but without an apparent cystic change, also showed significant reduction in VSV-G trafficking to the cell surface (column labeled “Normal” in Fig. 13C).

In a manner consistent with the observed mitochondrial fragmentation in cystic cells (Fig. 10), cystic cells displayed reduced mitochondrial function as assayed by uptake of TMRE (Fig. 13, D and E).

Protein partners that modulate the cystic ER phenotype. Several proteins have been shown to be involved in determin-
ing the structure of the ER. These include the reticulons (four members), the ATLs (three members), and the DP1/Yop1p proteins (six members) (2, 9, 19, 27–29, 36). In primary HPAECs, we found the major endogenous ATL to be ATL3 (Fig. 6B), with, at best, only low levels of ATL1 or ATL2. siRNA-produced knockdown of RTN4 over 4 days (which produced 70–80% reduction, as assayed by immunofluorescence) had minimal effect on development of the overall cystic phenotype, except that there was reduced nuclear distortion (Fig. 14A). Inclusion of ATL3 siRNA, together with STAT5a/b siRNA in respective transfections, led to partial effacement of cyst-zone boundaries (Fig. 14B). In magnetic-bead immunopanning assays, anti-ATL3 PAb, but not anti-ATL1, cross-bound both STAT5a and STAT5b (Fig. 4D).

Implications of the present findings in human disease. The cystic phenotype due to STAT5a/b knockdown reported here in primary human pulmonary arterial vascular cells, especially in Fig. 9B, was reminiscent of the EM findings of Smith and Heath (30, 31), who pointed to cystic dilatation of the ER in pulmonary arterial endothelial cells in hypoxic rats with pulmonary hypertension (PAH) [compare Figs. 4 and 8 in Smith and Heath (30) with the present Figs. 7D, 8, and 9Ba] and in cells in vascular lesions in a patient with pulmonary arterial hypertension [compare Fig. 8 in Smith and Heath (31) with the present Fig. 9Ba]. We, therefore, investigated whether the cystic ER/lunate nucleus/altered RTN4 phenotype might be observed in vascular cells in lung arterial lesions in patients with IPAH. Figure 15A shows that a cystic phenotype with
Lunate nuclear distortion was evident in cells in the proliferative and obliterative lesions in IPAH. Moreover, the proliferative arterial lesions included cells with increased RTN4 in the tunica media (Fig. 15B). A subset of these cells had a clear cystic phenotype, including increased RTN4, lunate distortion of the nucleus, and mislocalization STAT5a away from the nucleus (Fig. 15C; nuclei are greenish-blue, showing green STAT5a with blue 4,6-diamidino-2-phenylindole in left two

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Fig. 10. Fragmentation of mitochondria in the zones of cystic ER change. HPAECs transfected with the indicated siRNAs were fixed 1 day later and immunostained for mitochondria using anti-F1-ATPase monoclonal antibody (MAb) and for ER using anti-RTN4 goat PAb. The top row represents control cells transfected with scrambled siRNA. The middle row illustrates an example of an early cystic change due to STAT5a/b siRNA in which the mitochondria are relatively intact (single arrow). The middle and bottom rows illustrate examples of cystic change due to STAT5a/b siRNA in which mitochondria are fragmented (double arrows).

Fig. 11. The cystic ER phenotype of STAT5a/b−/− mouse embryo fibroblasts (MEFs). A: freshly plated early-passage STAT5a/b−/− and wt MEFs were imaged by phase-contrast microscopy 3 h after plating. Scale bar = 25 μm. B: live-cell cultures 6 h after plating were stained with ER-Tracker and imaged both in bright-field and by ER-Tracker fluorescence. ER cysts in the cell periphery were present in some STAT5a/b−/− MEFs (double arrows). Scale bar = 10 μm.
control cells, but largely clear blue showing reduced STAT5 with 4,6-diamidino-2-phenylindole in the right three PAH cells). Importantly, the combined knockdown of STAT5a and BMPRII (which is often mutated and/or haplo-insufficient in familial PAH; Refs. 20, 34) inhibited VSV-G trafficking to a greater extent than either siRNA alone (Fig. 15D). It is noteworthy that, in enumerating the data in Fig. 15D, all cells (i.e., both defined as “Normal” and “Cystic” in Fig. 13C) were included. In such experiments, cells without an overt cystic phenotype also showed reduced trafficking (data not shown).

DISCUSSION

The present study provides evidence for unexpected non-genomic functions of STAT5 species in the cytoplasm aimed at preserving the structure and function of the Golgi apparatus-ER unit. These data represent an instance in which a STAT “transcription factor” has now been implicated in preserving the structural integrity of a cytoplasmic organelle. STAT5 species, which transcriptionally upregulate expression of genes of bulk secreted proteins (e.g., milk), also preserve, non-transcriptionally, the integrity of the intracellular pathway in the cytoplasm required for synthesis and secretion of those proteins. The present data also highlight the occurrence of the cystic ER/lunate nuclear phenotype in cells in pulmonary vascular lesions in IPAH.

The technique of first washing cell cultures with a low-digitonin-isotonic sucrose buffer to remove bulk-soluble STAT proteins assisted in uncovering the association of endogenous STAT5a with the Golgi apparatus and centrosomes. This association was “constitutive” and did not require Tyr or Ser phosphorylation. In contrast, STAT5a associated with centrosomes was Ser phosphorylated. The constitutive association of STAT5a with the Golgi apparatus was also evident on transfection of cells with an exogenous expression vector for STAT5a-GFP. Mutants of STAT5a-GFP at either or both of the Ser or Tyr phosphorylation sites all exhibited Golgi association. Despite the extensive use of GFP-tagged proteins in localization studies at the Golgi apparatus and other cellular organelles (15), we are mindful that interpretation of the specificity of such association is limited by the apparent association of the free N1-EGFP tag peptide with cytoplasmic structures (our data not shown).

The consequences of acute STAT5a/b knockdown on the structure and function of the ER-Golgi-mitochondrial axis
Fig. 13. Functional consequences of STAT5a/b knockdown.
A–C: STAT5a/b knockdown inhibits intracellular vesicular stomatitis virus G protein (VSV-G) trafficking. EA.hy926 endothelial cells were transfected with an expression construct for tsO45 VSV-G-GFP, together with the respective siRNAs, and kept overnight at 39.5°C. The cultures were then shifted to 32°C for 1 h or kept at 39.5°C, then shifted to 4°C, and the intact cultures treated in the cold for 10 min with a MAb to a VSV-G epitope, which is exposed on the cell-surface (8G5F11). A: the cultures were then fixed and imaged for GFP (in green) and 8G5F11 MAb (in red). None of the cells kept at 39.5°C showed cell-surface VSV-G (data not shown). B and C: quantitation of VSV-G trafficking was pooled from four replications expressed as means ± SE; n = no. of cells enumerated. *P < 0.01.

D and E: STAT5a/b knockdown inhibits mitochondrial function. HPAECs transfected with siRNA, as indicated, were exposed 1 day later to tetramethylrhodamine ethyl ester (TMRE; 5 nM) for 15 min at 37°C and washed, and TMRE uptake was imaged by fluorescence. Data are from two independent replications; n = no. of cells enumerated, with uptake expressed as mean ± SE. *P < 0.01.
were unexpected. Cystic dilatation of the ER, dilatation and fragmentation of the Golgi cisternae, lunate distortion of the nucleus, and mitochondrial fragmentation in cystic ER zones was a dramatic phenotypic change elicited in several different cell types on acute knockdown of STAT5a/b. There was a marked tubule-to-cyst or tubule-to-sheet-to-cyst change in the ER. RTN4 accumulated at boundaries of cystic change and on the convex sides of distorted nuclei. We believe that the overt cystic-cell phenotype represents an extreme consequence of acute STAT5a or STAT5b knockdown one at a time, occurring in a minority subset of HPAECs in a given culture, even though the majority of cells in such cultures showed marked reduction in STAT5, as assayed by immunofluorescence. In contrast, in cultures transfected with both STAT5a and STAT5b siRNAs, up to 50–70% of the surviving cells showed an overt cystic phenotype.

The STAT5a/b-siRNA-knockdown phenotype developed in the presence of the mRNA synthesis inhibitor DRB. Thus this phenotype was the result of nongenomic effects, resulting from the reduction in STAT5a/b. While other investigators have reported nongenomic cytoplasmic functions of STAT3 at endosomes, mitochondria, focal adhesions, microtubules, and mitotic spindle (6, 21, 37), the observation that STAT5a/b knockdown altered the structure of cytoplasmic organelles, the ER and Golgi in the present instance, is without precedent. Off-target effects of the particular STAT5a/b siRNAs used in the present study (see source and catalog numbers in MATERIALS AND METHODS) are unlikely to contribute to the present observations in that 1) the human-specific STAT5a/b siRNAs did not elicit a cystic phenotype in MEFs, although murine-specific siRNAs did; and 2) STAT5a/b−/− null MEFs displayed altered ER/Golgi dynamics. Additionally, Hosui et al (8a) have reported the spontaneous development of a vacuolated phenotype in hepatocytes in mice in which the STAT5a/b locus had been homozygously deleted in a liver-specific manner compared to wt controls (Fig. 6A, left and center panels, in Ref. 8a). However, the presence of one allele of STAT5a/b even with an NH2-terminal truncation of 90 amino acids inhibited development of this phenotype (Fig. 6A, rightmost panel, in Ref. 8a). The vacuolation was interpreted by Hosui et al (8a) to repre-

Fig. 14. Effects of knockdown of the ER-resident proteins RTN4/Nogo-B or ATL3 on development of the STAT5a/b-siRNA-induced cystic phenotype. A: HPAECs were transfected with RTN4 siRNA or scrambled siRNA on day 0 and day 2, and then on day 4 the cultures were challenged with STAT5a/b siRNAs, fixed the next day, and the phenotype evaluated by phase-contrast microscopy (not shown) and immunostaining for GM130 and RTN4 and staining with DAPI. Arrows indicate cells with Golgi fragmentation and cystic phenotype in both the top and bottom rows. Scale bar = 5 μm. B: HPAECs were transfected with STAT5a/b siRNAs, together with either scrambled siRNA (a) or ATL3 siRNA (b). The cultures were fixed the next day and imaged by phase-contrast microscopy (not shown) and immunostaining for RTN4 and ATL3 and staining with DAPI. The bottom rows in Ba and Bb show higher magnification views of the cyst-zone boundaries, indicated by the boxed areas. Scale bars = 10 μm.
sent “fat droplets,” a known derivative of the ER, but the data presented (H&E light microscopy) are also consistent with cystic dilatation of the ER per se as in Fig. 15, A and C, in the present study.

The cyst-zone boundaries were demarcated by increased deposition of ATL3 and RTN4; these proteins were also observed along intercystic membranes. RTN4 preferentially accumulated on the convex sides of distorted nuclei. Knockdown of RTN4 did not affect development of the cystic phenotype, but appeared to blunt development of nuclear distortion. Thus the accumulation of RTN4 on the convex sides of nuclei likely participates in producing nuclear distortion. Partial knockdown of ATL3 reduced the demarcation of cyst-zone boundaries. Importantly, both endogenous STAT5a and STAT5b could be cross-immunopanned using an anti-ATL3 PAb, but not an anti-ATL1 PAb. Thus we anticipate that STAT5/ATL3 interactions may mechanistically underlie the observed phenotypic changes.

Compared with early passage wt MEFs, early passage STAT5a/b−/− null MEFs responded to the stress of plating by exhibiting a flagrant cystic ER phenotype, which was, however, transient and diminished by 12–24 h. Thus transience of the cystic ER phenotype observed in STAT5a/b−/− null MEFs suggests the existence of compensating mechanisms with respect to ER structure, as has been previously suggested to occur with respect to STAT signaling in STAT5a/b−/− null MEFs and mice (7, 16). Nevertheless, such STAT5a/b−/− null MEFs displayed persistent Golgi enlargement and fragmentation and megalocytosis.

Functionally, STAT5a/b knockdown resulted in cells that showed impaired membrane-protein (VSV-G) trafficking from the ER to the plasma membrane. It appears that this impairment encompassed multiple steps along the trafficking pathway, in that cells with VSV-G trapped in the ER, as well as cells with VSV-G trapped in the Golgi, were evident, with both kinds of cells showing the absence of VSV-G from the cell surface. Moreover, cells without an overt cystic phenotype also showed significant inhibition of VSV-G trafficking (Yang YM, Lee JE, Sehgal PB, unpublished observations). Additionally, STAT5a-siRNA knockdown inhibited trafficking of FLAG-

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Fig. 15. Prevalence of the increased RTN4/cystic ER/lunate nuclear distortion phenotype in vascular lesions of idiopathic pulmonary arterial hypertension (IPAH). A: hematoxylin and eosin (H&E) staining of human lung sections showing cystic cells in the proliferative and obliterative lesions in IPAH (IPAH-B; two right panels) compared with a normal arterial segment (Control-A) (H&E images of these lesions are in Fig. 1 in Ref. 25). B: increased expression of RTN4 in the pulmonary arterial wall in a proliferative lesion of IPAH (IPAH-B; right two panels; corresponds to lesion shown in A, center panel) compared with that in a control (Control-F, left two panels). The control and IPAH slides were processed at the same time, and images collected at identical settings. C: 2D compilations of z-stack imaging of single cystic cells with high-RTN4 with lunate nuclear distortion and mislocalized STAT5a in a proliferative lesion in IPAH (IPAH-B) compared with control arterial segments (Control-F). The IPAH-B images were from the lesion shown in A, center, and in B above. D: inhibition of VSV-G-GFP trafficking to the cell surface by the combination siRNA-mediated knockdowns of STAT5a and bone morphogenetic protein receptor type II (BMPRII). VSV-G-GFP trafficking to the cell surface was assayed as in Fig. 13A in EA.hy926 endothelial cells transfected 1 day earlier with respective siRNAs alone or in combination, as indicated. In this experiment, all cells were enumerated together without distinguishing between the cystic and noncystic phenotypes. Quantitation of VSV-G trafficking was pooled from four replications, expressed as mean ± SE; n = no. of cells enumerated. *P < 0.01 (Da and Db).
tagged wt BMPRII from ER to the cell surface in endothelial cells, even in those without an overt cystic phenotype (Yang YM, Lee JE, Sehgal PB, unpublished observations).

Development of the cystic phenotype was accompanied by reduced mitochondrial function (TMRE uptake), consistent with the observed mitochondrial fragmentation in regions of cystic change. The mitochondrial fragmentation in regions of cystic ER emphasizes the close interplay between ER tubules and the regulation of mitochondrial fission (Ref. 4 and citations therein). More generally, there appears to be an interdependence in the regulation of Golgi/ER/mitochondrial structure and function (4, 19, 15), an issue of particular significance in discussions of the pathogenesis of PAH (20, 32, 34).

Indeed, the present studies were carried out using primary HPAEC and HPASMC because of our long-standing interest in Golgi blockade in PAH (22). IPAH is a relentlessly fatal disease with a sexual dimorphism in that women are two- to fourfold more susceptible (20, 34). Histologically, lung vascular lesions in this disease have been reported to contain “plump” (31) and “vacuolated” (33) cells. Our laboratory previously reported Golgi enlargement and fragmentation and increased cytoplasmic dispersal of giantin in pulmonary vascular cells within IPAH lesions, accompanied by defects in intracellular trafficking (11, 12, 22, 24, 25). More recently, increased cellular levels of RTN4/Nogo-B have been reported in endothelial and smooth muscle cells in the proliferative arterial lesions of IPAH, and also that RTN4+/− mice failed to develop PAH in response to chronic hypoxia (32). These authors also identified the ATF6 ER-stress signaling pathway as mediating the increased expression of RTN4/Nogo-B in HPAECs and HPASMCs in patients with IPAH. Indeed, RTN4/Nogo-B has been implicated as a negative regulator of arterial remodeling and neointima development in a mouse model of mechanical femoral artery injury (1, 10). Other investigators have reported reduced mitochondrial function and increased mitochondrial fragmentation in cells derived from vascular lesions of IPAH (38).

Strikingly, the present EM data showing cystic ER dilatation in HPAECs upon STAT5a/b knockdown (Figs. 7C, 8, 9B) were reminiscent of the data of Smith and Heath (30, 31), showing cystic dilatation of the ER in cells in a pulmonary vascular lesion in a patient with PAH, as well as in PAECs in the hypoxic rat. The present findings of cystic cells with increased RTN4, lumen distortion of the nucleus, and cytoplasmically sequestered STAT5a in vascular lesions in IPAH suggest involvement of STAT5-related ER/Golgi mechanisms in the pathogenesis of this disease. The present observations provide a basis for considering the Golgi-ER-mitochondrial dysfunctions in cells in lesions in IPAH as representing a coordinated set of subcellular organellar alterations involving the Golgi apparatus, ER, and mitochondria.

Given the low penetrance (10–15%) of BMPRII mutations/deletions in causing overt disease, even in familial PAH, investigators have searched extensively for candidate “second-hit” genes (20, 34). The observation that VSV-G trafficking was significantly inhibited upon combining the functional haploinsufficiencies of STAT5a and BMPRII suggests that STAT5a has the potential for being such a “second-hit” gene. Moreover, it is noteworthy that STAT5 species are known to be estrogen and prolactin responsive (3, 5, 7, 16, 17, 35), and thus STAT5 biology in the cytoplasm might well underlie sexual dimorphic features of IPAH (higher prevalence in women) (20, 34).

To summarize, the present study provides novel evidence for nongenomic functions of STAT5 species in the cytoplasm aimed at preserving the structure and function of the Golgi apparatus-ER unit. The present data also highlight the occurrence of the cystic ER/lunate nuclear phenotype in cells in pulmonary vascular lesions in a relentlessly fatal human disease: IPAH.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


