Exon 4-encoded sequence is a major determinant of cytotoxicity of apolipoprotein L1

Atanu K. Khatua,1 Amber M. Cheatham,1 Etty D. Kruzel,2 Pravin C. Singhal,3 Karl Skorecki,2 and Waldemar Popik1
1Meharry Medical College, Center for AIDS Health Disparities Research, Nashville, Tennessee; 2Rappaport Faculty of Medicine and Research Institute, Technion-Israel Institute of Technology, Rambam Medical Center, Haifa, Israel; and 3Feinstein Institute for Medical Research, Manhasset, New York

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Khatua AK, Cheatham AM, Kruzel ED, Singhal PC, Skorecki K, Popik W. Exon 4-encoded sequence is a major determinant of cytotoxicity of apolipoprotein L1. Am J Physiol Cell Physiol 309: C22–C37, 2015. First published April 29, 2015; doi:10.1152/ajpcell.00384.2014.—The apolipoprotein L1 (APOL1) gene (APOL1) product is toxic to kidney cells, and its G1 and G2 alleles are strongly associated with increased risk for kidney disease progression in African Americans. Variable penetrance of the G1 and G2 risk alleles highlights the significance of additional factors that trigger or modify the progression of disease. In this regard, the effect of alternative splicing in the absence or presence of G1 or G2 alleles is unknown. In this study we investigated whether alternative splicing of non-G1, non-G2 APOL1 (APOL1 G0) affects its biological activity. Among seven APOL1 exons, exons 2 and 4 are differentially expressed in major transcripts. We found that, in contrast to APOL1 splice variants B3 or C, variants A and B1 demonstrate strong toxicity in human embryonic kidney (HEK293T) cells. Subsequently, we established that exon 4 is a major determinant of toxicity of variants A and B1 and that extracellular release of these variants is dispensable for their cytotoxicity. Although only variants A and B1 induced nuclear translocation of the transcription factor EB (TFEB), a master regulator of lysosomal biogenesis and autophagy, exon 4-positive and -negative APOL1 variants stimulated perinuclear accumulation of unprocessed autophagosomes. Knockdown of endogenous TFEB did not attenuate APOL1 cytotoxicity, indicating that nuclear translocation of TFEB is dispensable for APOL1 toxicity. Our findings that a human podocyte cell line expresses exon 4-positive and -negative APOL1 transcripts suggest that these variants may play a differential role in podocyte pathology. In summary, we have identified exon 4 as a major determinant of APOL1 G0 cytotoxicity.

apolipoprotein L1; autophagy; endocytosis; cytotoxicity; RNA splicing

Two coding variants of the apolipoprotein L1 (APOL1) gene (APOL1) have been recently attributed to the increased risk of developing nondiabetic kidney disease in individuals of recent African ancestry (10, 46). These risk variants, termed G1 (S342G and I384M substitutions) and G2 (N388 and Y389 deletions), localize in the COOH terminus of the protein encoded by exon 7 and have risen to high frequency in sub-Saharan African populations by providing resistance against the blood parasite Trypanosoma brucei rhodesiense and, possibly, other pathogens (32, 47). Although one APOL1 risk allele is usually sufficient to protect against this parasitic infection, two copies of any of the APOL1 risk alleles predispose the carriers to kidney disease. Because functional APOL1 protein is found only in humans, gorillas, and baboons (42), it has been suggested that APOL1 is dispensable for normal kidney function but may have an auxiliary function. Indeed, a nonessential role for APOL1 in humans has been confirmed by identification of individuals that carry homozygous APOL1 null alleles without detectable deleterious effects on kidney function (17). Although the functions of no-risk variant [wild-type (G0)] APOL1 proteins remain unknown, recent studies using in vitro (26, 34) and in vivo (45) models have demonstrated increased cytotoxicity of APOL1 G1 or G2 alleles compared with the APOL1 G0 allele. However, the overall disease penetrance, even with two risk alleles, is low, suggesting the importance of epistatic and environmental interaction effects (4, 6, 34). The most prominent example is HIV infection, which, when untreated, increases the risk of progressive kidney disease in individuals with two APOL1 risk alleles from ~4% lifetime risk to >50% lifetime risk (24). Importantly, this form of HIV-associated kidney disease [HIV-associated nephropathy (HIVAN)] is virtually absent in HIV-infected individuals without APOL1 risk alleles (3).

Alternative splicing of mRNA is a highly regulated process that increases proteome complexity by synthesis of proteins often with novel biological functions (21). Thus, epistatic or environmental factors may modify the APOL1 splicing pattern, resulting in alternatively spliced variants with potentially different pathobiological effects. The APOL1 gene is encoded by seven exons (8), which can be differentially spliced, usually by exclusion of exon 2 or 4, to encode three major APOL1 splice variants, A, B, and C (Fig. 1). Although the prevalence of different APOL1 splice variants is unknown, the most common, variant A, is encoded by exons 1 and 3–7. The splice variant B1 is encoded by exons 1–7, while variant B3 was reported to lack exon 4, as reported in an abstract presented in 2014 at the 10th International Podocyte Conference in Freiburg, Germany (48). Variant C is similar to variant A but lacks exon 4 and is encoded by exons 1, 3, and 5–7. With the exception of untranslated exon 1, all exons are fully or partly translated. Since the G1 and G2 alleles are located in the last and the largest exon 7, all APOL1 splicing variants will include these mutations, if present. However, the function of APOL1 proteins encoded by alternatively spliced APOL1 variants in the absence or presence of G1 or G2 risk alleles is unknown. Interestingly, exons 2, 3, and 4 contribute to the putative NH2-terminal signal peptide involved in endoplasmic reticulum targeting, membrane trafficking, and protein secretion. Thus it is likely that APOL1 variants B3 and C, which do not carry a canonical signal peptide because of the absence of exon 4, may display different intracellular localization, processing,
and function. Indeed, it was recently reported that APOL1 variant B3 localizes to mitochondria, although its function there was not determined (48). We previously showed that APOL1 splice variant A stimulates endocytosis and lysosomal biogenesis by promoting nuclear localization of transcription factor EB (TFEB) and expression of several TFEB target genes (44), suggesting that APOL1-mediated abnormalities in the endolysosomal pathway may contribute to aberrant autophagy and toxicity (26). Interestingly, the proinflammatory cytokines IFNγ and TNFα, known to potently stimulate expression of a major APOL1 variant, variant A (33, 34, 51), also increase the levels of alternatively spliced variants B and C (34). However, the pathological significance of these observations is unclear. In the present study we examined the effects of different APOL280(732,730),(952,760) variants on cell toxicity and downstream pathways including autophagy. We have found that exon 4 is a major determinant of cytotoxicity of APOL1 splice variants A and B1 but is dispensable for the inhibition of autophagosome maturation demonstrated by all tested APOL1 splice variants. We propose that exon 4 is a major determinant of APOL1 toxicity, an effect that could potentially be fortified by G1 or G2 risk alleles. Importantly, we showed that a human podocyte cell line expresses exon 4-positive and -negative APOL1 transcripts.

MATERIALS AND METHODS

APOL1 Expression Vectors and Mutagenesis

APOL1 cDNA corresponding to a splice variant A (NM_003661) was obtained in pcMV6-XLS vector (Origene). All APOL1 variants were expressed from pCMV6-XL5. APOL1 variant B1 (NM_145343, Geneart, Life Technologies) was PCR-amplified from a provided vector using primers containing EcoRI and XhoI restriction sites (underlined): 5’ A TCG GAA TTC GCC ATG AGA TCC AAC AGC CAC 3’ (forward EcoRI primer) and 5’ A GAT TCT AGA TCA CAG TTC TTG TTC CTG CC 3’ (reverse XhoI primer). The PCR product corresponding to full-length APOL1 cDNA was cloned into EcoRI and XhoI sites of pCMV6-XL5. The open reading frame of APOL1 genes was modified by PCR with myc epitope (MASMQKLISEEDL) separated from the APOL1 COOH-terminal sequence QADQEL with a linker composed of two amino acid residues proline and glycine. Myc epitope and APOL1 deletion mutants were created using the QuickChange II site-directed mutagenesis kit (Stratagene). APOL1 variant C (NM_001136541) was generated by deletion of amino acids 32–49 (corresponding to a fragment encoded by exon 4) from APOL1 variant A using a set of two complementary primers: 5’-G GGG 3’ and 3’-GAT GCA GAG GAC AGA GAC TC 3’. The DNA sequences of the PCR primers used in the first PCR were sequence-verified. Sequences of the PCR primers used in the first PCR were designed to discriminate variants with and without exon 4.

Tissue Culture, Treatments, and DNA and siRNA Transfections

Human conditionally immortalized glomerular podocytes, AB8/13 (39), were cultured at 33°C in RPMI medium supplemented with 10% fetal calf serum (FCS), gentamicin (50 μg/ml), and insulin-transferin-selenium (ITS, Life Technologies). For differentiation of podocytes, cells were transferred to 37°C for 7–10 days to inactivate temperature-sensitive SV40 large T antigen. For induction of APOL1 expression, differentiated podocytes were treated for 24 h with IFNγ (50 ng/ml; Peprotech) (34). HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and gentamicin (50 μg/ml). The cells were transfected using polyethylenimine (PEI, 25,000 mol wt; Polysciences), PolyFect (Qiagen), or Lipofectamine (Life Technologies). Transfections were performed on cells seeded at 5 × 10^5 seeded per well and growing on collagen-coated six-well plates using a total of 2 μg of DNA per well. APOL1 plasmid DNA was used at 1 μg per transfection. Total DNA in transfections was balanced with pcDNA3.1. For siRNA transfections, HEK293T cells were seeded in six-well plates at 1.2 × 10^5 per well. Transfection with Silencer Select predesigned siRNAs against autophagy protein 5 (Atg5) siRNA, TFEB siRNA, or control siRNA (Life Technologies) was performed on day 1 and repeated on day 2 using 20 nM final concentration of each siRNA and Lipofectamine RNAiMAX (Life Technologies), according to the manufacturer’s protocol. After 24 h, the cells were transfected with pcDNA3.1 or APOL1 expression vector (44), and after an additional 24 h, cells were lysed in RIPA buffer (Santa Cruz Biotechnology), and an equal amount of lysate proteins (20–50 μg) was separated on 10% SDS-polyacrylamide gel and analyzed by immunoblotting for expression of APOL1 (HPA018885 antibody, Sigma-Aldrich), Atg5 (Cell Signalining), and actin (Sigma-Aldrich). For analysis of TFEB knockdown, total cellular RNA was isolated using the RNeasy mini kit (Qiagen), and cDNA was generated from 1 μg of RNA using the iScript cDNA synthesis kit (Bio-Rad). Expression of TFEB, APOL1, and GAPDH RNAs was analyzed by real-time PCR (CFX96, Bio-Rad) using sets of specific primers (Origene). TFEB and APOL1 RNA levels were normalized against GAPDH RNA.

Analysis of APOL1 Splice Variant Transcripts in Human Podocytes

AB8/13 podocytes differentiated for 7 days in six-well plates were left untreated (control) or treated with IFNγ (50 ng/ml). After 24 h, total RNA was isolated (Qiagen), and 1 μg of RNA was reverse-transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad). cDNA volume was adjusted with nuclease-free water to 150 μl, and 3 μl of cDNA were subjected to a two-step PCR using Platinum Taq DNA polymerase (Life Technologies) in a final volume of 25 μl. PCR cycling conditions for the first and second PCRs were as follows: 95°C for 1 min followed by 18 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Primers for the first PCR were designed to anneal to exons 1 and 6 and to amplify fragments of all major splice variants of APOL1. Sequences of the PCR primers for the first PCR were as follows: 5’-GAAGAAAGCTGGATCTTGTCAG-3’ (exon 1 forward) and 5’-GGTCCAGGCTTATTACATTGTCAG-3’ (exon 6 reverse). In the second PCR, 1 μl of the first PCR product was amplified in a final volume of 25 μl using conditions described for the first PCR and primer sets designed to discriminate amplification fragments with and without exon 4. Sequences of primers used in the second PCR were as follows: 5’-GGCTCTTGGTACTATAGG-3’ (exon 3 forward) and 5’-GATGACGACTTATAGGTC-3’ (exon 3 reverse). The predicted size of the second PCR product for exon 4-negative APOL1 is 196 bp and that for exon 4-containing APOL1 is 250 bp.

PCR products were resolved on 3% agarose gel and visualized with ethidium bromide. DNA bands were excised, and DNA was extracted using the QIAquick gel extraction kit (Qiagen). Isolated DNA was cloned directly into the pJET vector using the CloneJET PCR cloning kit (Thermo Scientific), and ligation products were transformed into MAX Efficiency DH5α competent cells (Life Technologies). Plasmid DNAs were isolated from bacterial colonies using the QIAprep spin miniprep kit (Qiagen), and PCR DNA inserts were sequenced using sequencing primers provided with the CloneJET kit. The sensitivity and specificity of the PCR primers used to amplify cDNA from podocytes were initially tested on cDNA prepared from HEK293T cells transfected with DNA plasmids expressing APOL1 splice variants. PCR primers amplified products with predicted sizes.

Cytotoxicity Assays

Lactate dehydrogenase assay. HEK293T cells, cultured on six-well plates, were transfected with 1 μg each of control pcDNA or indicated
APOL1 constructs using PolyFect (Qiagen). After 5 h, the media were replaced with DMEM containing 2% FCS, and the cells were incubated overnight. Where indicated, culture media were supplemented with 50 μM chloroquine diphosphate (CLQ; Sigma-Aldrich), a lysosomotropic agent that prevents lysosomal acidification and inhibits function of lysosomal enzymes. After 24 h, culture media were harvested and centrifuged for 15 min at low speed to remove cell debris, and lactate dehydrogenase (LDH) activity was quantitated using a Cytoscan-LDH cytotoxicity assay (G-Biosciences) following the manufacturer’s protocol. Enzyme activity of LDH was assayed quantitatively by measurement of absorbance at 490 nm using a microplate reader. Cytotoxicity was expressed as a percentage of LDH released in experimental samples (subtracted for LDH present in 2% FCS culture medium) relative to LDH released by totally lysed cells (set for 100%).

Propidium iodide and Hoechst 33342 staining. Cells were incubated for 10 min at 37°C with fresh culture media supplemented with 1 μM Hoechst 33342 stain and 5 μM propidium iodide (PI; Life Technologies), washed twice with PBS, and fixed for 15 min in 4% paraformaldehyde (Sigma-Aldrich). The cells were washed with PBS and then imaged by fluorescence microscopy.

Autophagy Assays

Monitoring autophagy by fluorescence microscopy. HEK293T cells were transfected with GFP-microtubule-associated protein 1 light chain 3 (LC3) or GFP-LC3 ΔG (GFP-LC3 G120A) (43), a negative control defective in conjugation with phosphatidylethanolamine (PE), in combination with vectors expressing APOL1 mutants or splice variants. The cells were cultured for 24 h in nonstarving conditions (10% FCS), and formation of GFP fluorescent puncta (corresponding to autophagosomes) was observed under fluorescence microscopy. In cells transfected with GFP-LC3, only infrequent GFP puncta were detected in the cytoplasm, and most GFP-LC3 localized to the nucleus.

Analysis of autophagosome maturation. Autophagosome maturation was analyzed by confocal microscopy in cells transfected with a tandem fluorescent monomeric red fluorescent protein (mRFP)-enhanced GFP (EGFP)-LC3 construct (tfLC3). Because mRFP is more stable than EGFP in acidic conditions, effective fusion of autophagosomes with lysosomes can be traced by accumulation of red-fluorescent puncta (autolysosomes) as opposed to yellow puncta, which mark unprocessed autophagosomes (or fusion intermediates with reduced acidification).

Biochemical assays to monitor accumulation of lipiddated GFP-LC3 II. Lysates from cells transfected with GFP-LC3 and pcDNA3.1 (control) or APOL1 expression vectors were resolved on 10% SDS-polyacrylamide gel, and faster-migrating PE-conjugated GFP-LC3 II (which labels autophagosomes) and slower-migrating cytoplasmic GFP-LC3 I were separated and detected by immunoblotting using anti-GFP antibody (Santa Cruz Biotechnology).

Analysis of autophagic flux. Autophagic flux was analyzed 24 h after transfection by exposure of the cells for 3 h to 100 μM CLQ (11) (Sigma-Aldrich), which is known to inhibit lysosome acidification and fusion with autophagosomes. As a result, accumulation of unprocessed autophagosomes could be monitored by fluorescence microscopy (GFP-LC3 puncta) or by immunoblotting (GFP-LC3 II).

Permeabilization of Cells With Saponin

At 24 h after transfection, HEK293T cells were rinsed twice with PBS and permeabilized with 0.1% saponin (Sigma-Aldrich) in PBS at room temperature for a total of 10 min (two 5-min washes with PBS-saponin). After removal of saponin, the cells were lysed in RIPA lysis buffer, protein lysates were clarified by centrifugation, and equal amounts of protein (20 μg/lane) from control and saponin-treated cells were separated on 10% SDS-polyacrylamide gel (44) and analyzed for expression of APOL1, GAPDH, and actin (Sigma-Aldrich).

Endocytosis Assays

Transferin-Alexa Fluor 594 uptake. HEK293T cells were incubated for 18 h in a complete medium with 80 μM dynasore (Tocris Bioscience) (29) in 0.1% DMSO or 0.1% DMSO alone and then for 30 min at 37°C with 5 μg/ml transferin-Alexa Fluor 594 (Life Technologies). The cells were washed and then fixed in 4% paraformaldehyde for 15 min, washed in PBS, and imaged on a fluorescence microscope.

DQ Green BSA uptake. The cells pretreated with dynasore as described above were incubated for 4 h at 37°C with 250 μg/ml DQ Green BSA (self-quenched fluorescent dye BODIPY FL derivatives of BSA; Life Technologies) and then chased for 90 min in media without DQ Green BSA. DQ Green BSA acquires fluorescence only

Fig. 1. Apolipoprotein L1 (APOL1) splice variant schematics, terminology, and expression. The major APOL1 splice variants are cataloged in the National Center for Biotechnology Information (NCBI) database. Splice variant A (v.A) is encoded by transcript variant 1 (NM_003661) and contains exons 1 and 3–7 (protein composed of 398 amino acids (aa)); variant B1 (v.B1) is encoded by transcript variant 2 (NM_145343) and contains exons 1–7 (414 aa); variant B3 (v.B3) is similar to variant B1 but lacks exons 4 (396 aa); variant C is encoded by transcript variant 4 (NM_00136541) and contains exons 1, 3, and 5–7 (380 aa); A: major APOL1 variants encoded by different transcript splice variants. Amino acid numbering is shown as follows: the first translated amino acid is assigned the number 1 in the B1 variant, and amino acids for all splice variants, as well as engineered constructs, are then assigned numbers in relation to this amino acid. Thus the first amino acid number in variant A is 17. According to the published NCBI and Ensembl data, aa 10 (Leu) is encoded by the 2 last nucleotides of exon 2 (TT) and the first nucleotide (G) of exon 3; aa 31 (Trp) is encoded by the last TG (exon 3) and first G (exon 4); aa 49 (Arg) is encoded by the last AG (exon 4) and first G (exon 5); aa 79 (Glu) is encoded by the last G (exon 5) and first AG (exon 6); aa 121 (Arg) is encoded by the last AG (exon 6) and first G (exon 7). Untranslated exons or parts of exons are symbolized by white boxes; exons encoding for a putative signal peptide are shown in green and translated exons in blue. Exons (boxes) and introns (horizontal lines) are shown not in scale. B: APOL1 splice variants A, B1, B3, and C were constructed from APOL1 cDNA and, thus, include only the indicated exons without introns. Deletion mutants of variants A and B1 are also shown. Deletion of aa 17–43 from APOL1 variant A, denoted v.A(d.17–43), creates APOL1 variant A without a signal peptide. Deletion of aa 1–16 from variant B1 [v.B1(d.1–16)] produces variant A. Deletion of aa 32–49 from variant B1 produces v.B1(d.32–49), which is identical to variant B3. Deletion of aa 1–16 from v.B1(d.32–49) produces v.B1(d.1–16, 32–49), similar to variant C. APOL1 variant C was created by deletion of aa 32–49 from original variant A [denoted v.A(d.32–49)] and also by deletion of aa 1–16 and 32–49 from the original APOL1 variant B1 [denoted v.B1(d.1–16, 32–49)]. Similarly, in addition to the original variant A, a variant with exon composition identical to that of variant A was created by deletion of aa 1–16 from variant B1 and is denoted v.B1(d.1–16). C: amino acid sequences encoded by exons 2–7 of APOL1. All exons are translated only in APOL1 variant B1. Alternating exons are shown in blue and black. Amino acid residues shown in red are encoded by nucleotides from neighboring exons. D: APOL1 splice variants were transiently expressed in HEK293T cells and analyzed by Western blotting. APOL1 v.A(d.17–43) and variant B1 migrate in a SDS-polyacrylamide gel as proteins with apparent molecular size expected for APOL1 variant A. APOL1 v.B1(d.32–49) (same as variant B3) migrates as a doublet, which may result from posttranslational modification(s) or proteolytic processing, as suggested elsewhere (13). E: aberrant migration of the APOL1 proteins in a SDS-polyacrylamide gel does not result from proteolytic processing at the COOH terminus.
after dequenching through proteolytic enzyme cleavage of BSA and, thus, allows visualization of the endolysosomal compartment with proteolytic activity. The cells were washed with PBS and then fixed and imaged on a fluorescence microscope.

**Confocal Microscopy**

HEK293T cells were transfected on six-well plates using PolyFect; at 5 h after transfection, the cells were trypsinized, and a fraction of the cells was seeded in two-well chamber slides (Lab-Tek II) precoated with collagen. After overnight incubation, the cells were rinsed once with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were washed three times with PBS and then mounted with SlowFade antifade reagent with DAPI (Life Technologies) and observed under a laser scanning confocal microscope (Nikon TE2000).

**APOL1 Immunoprecipitation and Immunoblotting**

HEK293T cells were transfected on six-well plates with myc-tagged APOL1 expression constructs using PolyFect. At 5 h after transfection, media were replaced with DMEM containing 2% FCS. After overnight incubation, culture supernatants (2 ml each) were collected by low-speed centrifugation, filtered through 0.45-

**Additional Reagents**

Expression vectors GFP-LC3, GFP-LC3 ΔG, mRFP-EGFP-LC3, and GFP-TFEB were purchased from Addgene. Benzyloxy carbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-FMK) was purchased from Tocris Bioscience.

**Statistical Analysis**

All experiments were repeated at least three times. For comparison of mean values between two groups, a Student’s t-test was performed. P < 0.05 was considered statistically significant.

**RESULTS**

**The NH2-Terminal Fragment of APOL1 Encoded by Exon 4 Determines APOL1 Cytotoxicity**

Among the family of human APOL proteins, only APOL1 has the putative NH2-terminal signal peptide (35). Three major APOL1 protein splice variants, A, B, and C, are encoded by transcripts generated by different splicing events that involve the small exons 1–4, with exons 5–7 present in all transcripts (Fig. 1A). As a result, APOL1 splice variants differ only in the NH2-terminal region, which may affect the integrity of the signal peptide. To investigate whether such modifications may lead to different cellular functions of APOL1 splice variants, we have generated APOL1 expression vectors encoding several splice variants of APOL1 (Fig. 1B). An amino acid sequence encoded by APOL1 exons is presented in Fig. 1C. Noticeably, APOL1 amino acids 10, 31, 49, 79, and 121 are encoded by nucleotides from neighboring exons (Fig. 1C, shown in red). Expression of the APOL1 splice variants, tagged with c-myc at the COOH terminus, was confirmed by Western blot analysis using anti-APOL1 and anti-myc antibodies (Fig. 1, D and E). We have noticed that APOL1 variant A(d.17–43) and B1 migrate in a SDS-polyacrylamide gel with apparent molecular size expected for APOL1 variant A. In addition, APOL1 variant B3 migrates as a doublet, which may result from posttranslational modifications or partial proteolytic processing, since DNA sequence analysis of the expression vector did not reveal contamination with another APOL1 variant.

Although increased cytotoxicity of the risk variants G1 and G2 in the background of the most-studied variant A was reported, cytotoxicity of the wild-type APOL1 G0 variant was also observed (26, 34, 45). To investigate cytotoxicity of APOL1 splice variants in the absence of G1 or G2 risk mutations, these variants were overexpressed in HEK293T cells, which we found to be highly sensitive to toxic effects of APOL1. Cells undergoing apoptosis and dead cells were stained simultaneously with the DNA dyes Hoechst 33342 and PI (12). Cell-permeable Hoechst 33342 stains condensed nuclear DNA characteristic for apoptotic cells more brightly than normal chromatin (1). In contrast, a nonpermeant PI stains only cells with compromised plasma membranes or dead cells. While cells transfected with control pcDNA3.1 vector show only a few bright Hoechst/PI-stained cells in the examined area, expression of APOL1 splice variant A or B1 was highly cytotoxic, as shown by a loss of Hoechst-positive cells and an increase in cells brightly stained with Hoechst and PI (Fig. 2A). Deletion of the first 16 amino acid residues from variant B1, which creates a variant B1(d.1–16) similar to variant A, did not affect cytotoxicity, suggesting that amino acids 1–16 (encoded by exon 2 and part of exon 3) do not contribute to cytotoxicity of variant B1. Deletion of an additional fragment, amino acids 32–49, which is encoded by exon 4, resulted in APOL1 variant B(d.1–16, d.32–49), which shows strongly reduced cytotoxicity. Similarly, APOL1 splice variants C and B3, which lack the entire amino acid 32–49 region due to the absence of exon 4 in their transcripts, were not cytotoxic. These observations suggest that the region encoded by exon 4 is critical for APOL1 cytotoxicity, while the region between amino acid residues 1 and 31, encoded by exons 2 and 3, does not contribute to cytotoxicity. In line with these findings, deletion of amino acid fragment 17–43 in APOL1 variant A strongly inhibited cytotoxicity, suggesting that an even shorter fragment, between amino acids 32 and 43, contributes to APOL1 toxicity.

To assess quantitatively cytotoxicity of APOL1 splice variants, we measured colorimetrically the activity of LDH, a cytosolic enzyme released into the culture medium from damaged cells (27). The LDH assay confirmed a potent, statistically significant (P < 0.001) cytotoxicity of APOL1 variants A and B1 and lack of significant toxic effects of variants B3 and C (Fig. 2B). Since APOL1 variants A and B1 show a strong cytotoxic effect and variant A stimulates accumulation of lysosomes when overexpressed in HEK293T cells (44), we considered the possibility that lysosome damage and release of lysosomal proteases may contribute to toxicity, as previously observed in podocytes (26). How-
ever, incubation of the transfected cells with the cell-permeable lysosomal protease inhibitors E64d (an inhibitor of cathepsins B, H, and L) and pepstatin A (an inhibitor of cathepsins D and E) (44) did not reduce toxicity ($P < 0.001$; Fig. 2B), suggesting that damage to lysosomes is not a primary cause for cytotoxic effects of APOL1 variants A and B1 in HEK293T cells. To confirm these results, we investigated the effect of CLQ, an inhibitor of lysosomal acidification, on cytotoxicity of APOL1 splice variants. Results show that inhibition of lysosomal acidification by CLQ resulted in accumulation of autophagosomes (Fig. 4A) but does not reduce cytotoxicity of APOL1 variants A and B1 ($P < 0.001$; Fig. 2C). Together, these results indicate that the amino acid sequence encoded by exon 4 contributes to the increased cytotoxicity of APOL1 variants A and B1 in a lysosome-independent manner.
APOL1 Splice Variants Expressing a Sequence Encoded by Exon 4 Promote Nuclear Translocation of TFEB-GFP

We previously showed that APOL1 variant A promotes nuclear translocation of TFEB, which stimulates expression of lysosomal genes and leads to lysosomal biogenesis (44). Since TFEB also stimulates expression of autophagy genes (41), nuclear localization of TFEB could serve as an indicator of sustained autophagy activation. To assess the ability of APOL1 splicing variants to promote nuclear localization of TFEB, TFEB-GFP was expressed in HEK293T cells in the absence or presence of APOL1 splice variants, and cellular localization of TFEB-GFP was monitored by fluorescence microscopy (Fig. 3A). In the absence of APOL1, TFEB-GFP localized primarily to the cytoplasm, and, on average, only in $9 \pm 6\%$ cells was TFEB-GFP detected in the nucleus or in the nucleus and cytoplasm. However, nuclear localization of TFEB-GFP was strongly enhanced by APOL1 variants A, B1, and B(d.1–16), with $\sim 80\%$ of cells showing nuclear localization of TFEB-GFP ($P < 0.005$; Fig. 3B). In contrast, APOL1 splice variants and deletion mutants lacking the region encoded by exon 4 [variant A(d.17–43), variant B3, and variant C] did not noticeably change the nuclear-cyttoplasmic distribution pattern of TFEB-GFP (Fig. 3B).

To investigate whether nuclear translocation of endogenous TFEB mediates cytotoxicity of APOL1 variants expressing the exon 4-encoded sequence, knockdown of endogenous TFEB in HEK293T cells using TFEB siRNA was followed by transfection of the cells with APOL1 splice variants. Despite $\sim 80\%$ knockdown of TFEB mRNA ($P < 0.005$; Fig. 3D), cytotoxicity of APOL1 variant A or C was not affected ($P < 0.005$; Fig. 3C). In conclusion, toxic effects of APOL1 are not mediated by nuclear localization of TFEB.

![Fig. 3. APOL1 splice variants expressing a sequence encoded by exon 4 promote nuclear translocation of transcription factor EB (TFEB). A: HEK293T cells were transfected with TFEB-GFP and APOL1 splice variants, deletion mutants, or pcDNA3.1 (control); after 24 h, cells were fixed with 4% paraformaldehyde and analyzed by fluorescence microscopy. Scale bars = 50 μm. B: nuclear-cytoplasmic distribution of TFEB-GFP in ~100 cells expressing TFEB-GFP and APOL1 splice variants. Values are means ± SD from 3 independent transfections. Cells transfected with exon 4-positive APOL1 variants show statistically significant higher nuclear localization of TFEB-GFP than cells expressing exon 4-negative APOL1 variants. *$P < 0.005$ vs. pcDNA3.1 (control). C: nuclear translocation of endogenous TFEB does not mediate APOL1 toxicity. HEK293T cells were transfected with TFEB siRNA or control siRNA (Co siRNA); after 48 h, cells were transfected with APOL1 splice variants or pcDNA3.1 (pcDNA). After an additional 18 h, culture supernatants were collected and assayed for lactate dehydrogenase (LDH) cytotoxicity. Values are means ± SD from 3 independent transfections. *$P < 0.005$ vs. Co siRNA + pcDNA. **$P < 0.005$ vs. Co siRNA + APOL1 v.A. ***$P < 0.005$ vs. Co siRNA + APOL1 v.C. D: total cellular RNA was reverse-transcribed and subjected to real-time PCR analysis using sets of specific TFEB and GAPDH primers. Expression of TFEB mRNA was normalized against GAPDH mRNA. Knockdown of TFEB mRNA is statistically significant. *$P < 0.005$ vs. Co siRNA + pcDNA3.1. **$P < 0.005$ vs. Co siRNA + TFEB siRNA + pcDNA. ***$P < 0.005$ vs. Co siRNA + TFEB siRNA + APOL1 v.A. 

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[Image of Figure 3: A: HEK293T cells transfected with TFEB-GFP and APOL1 splice variants; B: nuclear-cytoplasmic distribution of TFEB-GFP; C: nuclear translocation of endogenous TFEB; D: real-time PCR analysis of TFEB mRNA.]

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Functional autophagy is critically important for maintaining homeostasis of podocytes and kidney function (9, 11, 15). Since nuclear localization of TFEB induces genes controlling autophagosome biogenesis (41), we next investigated whether the pattern of TFEB activation by APOL1 splice variants correlates with the induction of autophagy in HEK293T cells under a nutrient-rich condition. Accumulation of autophagosomes in cells overexpressing APOL1 splice variants was monitored by formation of fluorescent puncta by transfected GFP-LC3, a specific marker of autophagosomes (18). In light of nuclear translocation of TFEB, we expected that variants A and B1 would induce stronger accumulation of autophagosomes than exon 4-negative splice variants. Surprisingly, all tested APOL1 splice variants stimulated accumulation of GFP-LC3 puncta, indicative of accumulation of autophagosomes (Fig. 4A). In contrast to cells treated with CLQ, which show accumulation of small GFP-LC3 puncta distributed throughout the cytoplasm, overexpression of APOL1 splice variants resulted in formation of large and mainly perinuclear GFP-LC3 aggregates. To rule out the possibility that formation of GFP-LC3 puncta resulted from overexpression and nonspecific aggregation of GFP-LC3, the cells were transfected with a mutant GFP-LC3 ΔG in which COOH-terminal glycine 120 was substituted with an alanine residue, resulting in a protein unable to conjugate with PE, a step required for LC3 modification and insertion into an autophagosomal membrane (43). Immunofluorescence microscopy showed lack of fluorescent puncta in cells transfected with GFP-LC3 ΔG in the absence or presence of APOL1 variant A or B1 (Fig. 4B). This observation was further confirmed by Western blotting, which shows formation of PE-conjugated GFP-LC3 (GFP-LC3 II) in cells transfected with GFP-LC3, but not with GFP-LC3 ΔG mutant (Fig. 4C). This indicates that GFP-LC3 puncta accumulating in the presence of APOL1 variants represent autophagosomes. Furthermore, siRNA-mediated depletion of endogenous protein Atg5, required for the formation of autophagosomes (30), suppressed accumulation of autophagosomes induced by APOL1 variant A or by an inhibitor of lysosomal acidification, CLQ, which blocks fusion between autophagosomes and lysosomes (5) (Fig. 4, D and E). Formation of large GFP-LC3 structures suggests that APOL1 may stimulate aggregation of autophagosomes, which could interfere with their fusion with lysosomes. Indeed, a standard flux assay (31) indicates no major differences in the accumulation of GFP-LC3 II in cells transfected with various APOL1 splice variants in the absence or presence of CLQ, suggesting ineffective autophagosome processing, rather than induction of autophagy (Fig. 4F). In conclusion, a sequence encoded by exon 4 is dispensable for stimulation of autophagosome accumulation by APOL1 splice variants.

APOL1 Splice Variants Inhibit Autophagosome Maturation

Enhanced accumulation of autophagosomes usually results from increased autophagosome formation, reduced fusion of autophagosomes with lysosomes, or decreased proteolytic activity of lysosomes (37). Previously, we showed that APOL1 variant A stimulates accumulation of enzymatically competent lysosomes (44), suggesting that decreased proteolytic activity of lysosomes is less likely to affect autophagosome accumulation. To investigate the possibility that accumulation of autophagosomes results from decreased fusion with lysosomes, we took advantage of a tandem fluorescent protein mRFP-EGFP-LC3, which demonstrates yellow fluorescence as a result of similar stability of mRFP and green fluorescent (EGFP) proteins at neutral pH of the cytoplasm or autophagosomes (23). Since fluorescence of the EGFP moiety in mRFP-EGFP-LC3 quenches in the acidic pH of lysosomes, while mRFP exhibits more stable fluorescence, effective fusion of autophagosomes with lysosomes can be tracked by the accumulation of red-fluorescing speckles. Confocal microscopy analysis shows predominant accumulation of yellow speckles in the perinuclear region, suggesting that all tested APOL1 splicing variants impair fusion between autophagosomes and lysosomes (Fig. 5). Consequently, excessive accumulation of unprocessed autophagosomes may lead to their aggregation. However, a precise molecular mechanism underlying the observed inhibition of autophagosome maturation by APOL1 remains to be determined. HEK293T cells transfected with pcDNA3.1 (control) show predominant accumulation of the mRFP-GFP-LC3, as previously reported for other cell types (7).

Secreted APOL1 Does Not Contribute to Cytotoxicity

Although APOL1 cytotoxicity is limited to splice variants that carry a sequence encoded by exon 4, inhibition of autophagosome maturation in HEK293T cells is a hallmark of all examined APOL1 variants. Thus, to test the possibility that extracellular secretion and subsequent endocytic uptake of those APOL1 variants with a signal peptide (contributed by exon 4) could lead to increased cytotoxicity (26), we investigated release of myc-tagged APOL1 variants into the culture medium of transfected HEK293T cells. Surprisingly, all APOL1 variants were detectably immunoprecipitated from culture media of transfected cells (Fig. 6A). While the levels of APOL1 variants A and B1 could be overestimated due to cell damage induced by these variants, detection of variants B3 and C in immunoprecipitates suggests that these noncytotoxic splice variants may be released through a different mechanism. To examine whether APOL1 splice variants differ in their mechanism underlying the observed inhibition of autophagosome maturation (HEK293T cells were transfected with PC3.1 (control) show predominant nuclear accumulation of the mRFP-GFP-LC3, as previously reported for other cell types (7).
h of treatment of cells with a noncytotoxic (80 μM) concentration of dynasore, a specific inhibitor of clathrin-mediated endocytosis (29), did not affect intracellular accumulation of APOL1 variant B1 or B3 (Fig. 6C), suggesting that released APOL1 is not effectively endocytosed or is rapidly degraded upon internalization through a clathrin-independent pathway in these cells. Importantly, cytotoxicity of these variants was not detectably changed by treatment with dynasore (P < 0.005; Fig. 6D), suggesting that, at least in HEK293T cells, clathrin-mediated endocytosis of APOL1 does not contribute to its toxicity. As expected, dynasore inhibited endocytosis of Alexa Fluor 594-tagged transferrin (22) and DQ Green BSA, which
shows green fluorescence only when BSA is degraded in a functional endolysosomal compartment (Fig. 6E).

A Human Podocyte Cell Line Expresses APOL1 Splice Variant Transcripts

Not much is known about expression of APOL1 splice variants in cells. To investigate if APOL1 splice variants are expressed in podocytes, we have analyzed the splicing pattern of APOL1 in human conditionally immortalized and differentiated podocytes, AB8/13 (39), untreated or treated with IFNγ, which is known to induce APOL1 expression (34, 51). A set of PCR primers designed to anneal to exons 1 and 6 were used to amplify several DNA products, with a major (350-bp) DNA product that corresponded to the size predicted for APOL1 variant A (Fig. 7A). Interestingly, the putative APOL1 transcripts were detected also in unstimulated cells. To evaluate the existence of APOL1 splice variant transcripts in an unbiased manner, we designed PCR primer pairs for use in a two-step PCR (Fig. 7B). In the first step, all APOL1 transcripts containing exons 1 and 6 were amplified in 18 cycles, and a 1/25th fraction of the product volume was subjected to a second round of PCR with a new set of PCR primers (annealing to exons 3 and 6), which would allow us to distinguish between exon 4-positive and -negative transcripts. Eighteen-cycle amplification in the second PCR produced two major DNA products corresponding to putative exon 4-negative transcripts (196 bp, variant C/variant B3) and 250-bp exon 4-positive transcripts (variant A/variant B1). The identity of these transcripts was confirmed after extraction of DNA from the gel, cloning into pJET vector, and DNA sequencing (Fig. 7D). Densitometric analysis (Fig. 7B) shows that exon 4-positive and -negative APOL1 transcripts increased ~1.6-fold after IFNγ induction. The level of exon 4-positive transcripts was ~17-fold higher than the level of exon 4-negative transcripts in uninduced and induced podocytes. Interestingly, despite the moderate (~1.6-fold) increase in APOL1 splice variant transcript levels in podocytes stimulated with IFNγ, APOL1 protein levels were induced >16-fold by IFNγ (Fig. 7C). This suggests that, in unstimulated podocytes, APOL1 transcripts may be sequestered in translationally inactive compartments (e.g., stress granules or processing bodies) (20), from which APOL1 mRNAs could be quickly mobilized for immediate translation upon podocyte stimulation.

DISCUSSION

Increased cytotoxicity of APOL1 G1 or G2 variants has been demonstrated to be associated with podocyte injury (26); however, it is unknown whether there are additional determinants that may affect APOL1 cytotoxicity. Indeed, low penetrance of G1- and G2-associated kidney disease (24), as well as noticeable cytotoxicity of overexpressed wild-type APOL1 G0 (26, 34), suggests the existence of modifying factors that may
Fig. 6. Secreted APOL1 does not contribute to cytotoxicity. A: APOL1-myc splice variant proteins are released into culture media of transfected HEK293T cells. At 5 h after transfection, cells were transferred into DMEM supplemented with 2% FCS. After overnight incubation, culture media were collected, filtered, and precleared with Protein A/G PLUS-agarose, and APOL1-myc was immunoprecipitated using anti-myc antibodies conjugated with agarose beads. Cell lysate proteins and immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by immunoblotting for expression of APOL1 and GAPDH. In addition to APOL1 proteins, nonspecific proteins (most likely derived from serum-containing media) were also detected in anti-myc immunoprecipitates (asterisk). B: intracellular APOL1 is refractory to extraction with the nonionic detergent saponin. Permeabilization of cell membranes with saponin depleted cytosolic GAPDH but retained actin. At 24 h after transfection, cells were permeabilized with 0.1% saponin for 10 min, and protein lysates from untreated (Control) or saponin-treated cells (+Saponin) were analyzed by immunoblotting for expression of APOL1, GAPDH, and actin. C: secreted APOL1 does not contribute to the intracellular pool of APOL1. Transfected cells were cultured in the presence of 80 μM dynasore or solvent (DMSO). After 24 h, cells were permeabilized with 0.1% saponin, and cell protein lysates were separated by SDS-PAGE and analyzed for expression of APOL1, GAPDH, and actin. D: secreted APOL1 does not contribute to cytotoxicity. Cells were transfected with cytotoxic and noncytotoxic APOL1 variants B1 and B3, respectively, and cultured in the absence or presence of 80 μM dynasore. After 24 h, cell culture media were collected and analyzed for released LDH. Values are means ± SD of 3 independent samples. Corresponding protein lysates were analyzed by immunoblotting for expression of APOL1 and actin (bottom). *P < 0.005 vs. pcDNA3.1. **P < 0.005 vs. pcDNA3.1/Dynasore. E: Dynasore inhibits endocytosis of transferrin and DQ Green BSA. Transfected cells were pretreated for 24 h with 80 μM dynasore (+Dynasore) or DMSO (−Dynasore). Then cells were exposed for 30 min to 5 μg/ml transferrin-Alexa Fluor 594 (Trf-AF594) or for 4 h to DQ Green BSA (250 μg/ml) and then chased for 90 min in media without DQ Green BSA. Cells were washed and then fixed with 4% paraformaldehyde, mounted in antifade regent with DAPI (to stain nuclei), and imaged on a fluorescence microscope. Scale bar = 20 μm.
Fig. 7. Expression of APOL1 splice variant transcripts and APOL1 proteins in a human podocyte cell line. Differentiated AB8/13 podocytes were left untreated (−) or treated (+) with IFNγ (50 ng/ml). After 24 h, total RNA and protein lysates were prepared. A: RNA was reverse-transcribed into cDNA and subjected to a 2-step PCR. The 1st step of the PCR was performed using a set of primers (black arrows) that anneal to exon 1 (forward) and exon 6 (reverse) to amplify APOL1 cDNA from different splice variants. Predicted sizes of PCR products corresponding to variants B1, B3, A, and C are shown. PCR products amplified in 18 cycles were not detectable on an agarose gel, but APOL1 amplification was detected by real-time PCR (not shown). In contrast, 30-cycle amplification resulted in detectable PCR products, with a major PCR product at 350 bp. These products were not amplified without a previous reverse-transcription reaction (RT−), suggesting that isolated RNA was not contaminated with cellular DNA (right). B: to amplify exon 4-negative APOL1 splice variants, a new set of primers was designed to anneal to exon 3 (forward) and exon 6 (reverse). One μl of the 1st PCR product obtained after 18-cycle amplification (A) was amplified in a 2nd PCR (18 cycles), and products were separated on 3% agarose gel with ethidium bromide. Two PCR products were detected: a major (~250-bp) band corresponding to the expected size of the amplified fragment of variants A and B (v.A/v.B1) and a minor (~196-bp) band corresponding to the expected size of variants C and B3 (v.C/v.B3). Numbers shown next to the DNA bands represent intensities of DNA signals relative to intensity of the 196-bp band in unstimulated cells (set as 1.0) and were obtained from densitometric scanning. C: expression of APOL1 proteins in differentiated AB8/13 podocytes after 24 h of exposure to IFNγ (50 ng/ml). Immunoblot analysis (independent, duplicate samples) shows strong induction of APOL1 proteins. As determined by densitometric scanning, podocytes stimulated with IFNγ express 16.1- to 17.3-fold higher levels of APOL1 proteins than unstimulated control cells. D: DNA sequencing of cloned 250- and 196-bp PCR products confirms expression of exon 4-positive and -negative APOL1 transcripts in differentiated human AB8/13 podocytes. Amino acid sequences of the exon 4-negative (top) and exon 4-positive (bottom) splice variants were determined from DNA sequences.
increase disease penetrance. The most striking example is development of HIVAN in HIV-1-infected individuals with two copies of APOL1 risk allele G1 or G2. Accordingly, the presence of risk alleles is the major contributor to development of HIVAN, which develops in >50% of G1 or G2 carriers. Interestingly, although HIVAN was not found in the HIV-1-infected Ethiopian population, which is known to lack APOL1 G1 or G2 risk alleles (3), Atta et al. (2) provide evidence that HIVAN may develop in some African American patients who do not carry APOL1 risk alleles. This observation suggests that, in specific conditions, APOL1 G1 or G2 risk alleles may be dispensable for development of HIVAN. However, whether APOL1 G0, with or without other genetic risk variants, can substitute for G1 or G2 in HIVAN is unknown. Because APOL1 G0 is also cytotoxic when overexpressed (49, 51), we have investigated the possibility of the existence of G1/G2-independent determinants that may contribute to APOL1 G0 toxicity.

Over 90% of human genes are alternatively spliced, indicating that alternative splicing of pre-mRNA represents a major mechanism for expanding gene function and proteome diversity (50). We thus investigated the possibility that alternatively spliced APOL1 G0 transcripts (reported by several databases, including the National Center for Biotechnology Information and Ensembl) contribute to G1/G2-independent toxicity of APOL1 G0. APOL1 can be expressed from several alternatively spliced mRNAs. The major APOL1 splice variants, A, B, and C, differ in their NH2-terminal amino acid composition and their homeostasis (11, 15).

To investigate whether alternatively spliced APOL1 transcripts can produce proteins with different biological activities, APOL1 proteins were expressed from engineered vectors encoding splice variants (Fig. 1B). Since increased cytotoxicity is a hallmark of APOL1 G1/G2 risk variants (26, 34, 45), we analyzed cytotoxicity of alternatively spliced APOL1 G0 variants in human kidney HEK293T cells. These cells were selected because of high transfection efficiency, lack of detectable levels of endogenous APOL1, and high sensitivity to the toxic effects of APOL1. Although use of this cell system proved to be useful in identifying differences in biological activities between APOL1 variant proteins, this experimental system suffers from some shortfalls. One is that overexpressed proteins may change or acquire additional behavior. However, expression levels of endogenous APOL1 in podocytes (Fig. 7C) were not significantly different from those detected in HEK293T cells transfected with limiting amounts of APOL1 expression vectors (Fig. 1D). However, we cannot exclude the possibility that overexpressed APOL1 may differently affect proliferating HEK293T cells and differentiated, nondividing podocytes, which strongly depend on autophagy to maintain their homeostasis (11, 15).

By staining transfected HEK293T cells with the DNA dyes Hoechst 33342 and PI (which stain apoptotic and necrotic cells, respectively), we found that APOL1 variants A and B1 demonstrate increased cytotoxicity compared with splice variants B3 and C, which lack the amino acid sequence 32–49 encoded by exon 4. Furthermore, deletion of the first 16 amino acid residues (encoded by exon 2 and part of exon 3) from variant B1 did not reduce cytotoxicity, suggesting that these exons do not contribute to the toxicity of APOL1. However, deletion of a sequence encoded by exon 3 and part of exon 4 (residues 17–43) from variant A inhibited a cytotoxic phenotype of variant A, indicating that exon 4 (or even its subfragment comprising residues 32–43) is a major determinant of APOL1 cytotoxicity. These findings were subsequently confirmed using a quantitative LDH cytotoxicity assay. In light of our previous findings showing increased lysosomal abundance in HEK293T cells (44) and increased lysosomal membrane permeability in podocytes expressing APOL1 (26), we expected to observe reduced APOL1 cytotoxicity in the presence of inhibitors of lysosomal proteases or inhibitors of lysosomal acidification (CLQ). However, lack of the effect of these compounds indicates that, in contrast to podocytes (26), APOL1 toxicity in HEK293T cells is not mediated by excessive lysosomal damage.

TFEB controls multiple steps of the autophagy pathway and may ensure prolonged activation of autophagy, which is generally considered a cytoprotective pathway under conditions of stress. TFEB is normally localized to the cytosol but translocates to the nucleus under starvation conditions or when lysosomal function is impaired. Such translocation enables the coordinated expression of several genes belonging to the Coordinated Lysosomal Expression and Regulation (CLEAR) network, which controls lysosomal biogenesis and autophagy (36, 40, 41), processes that are critical for the homeostasis of long-lived and terminally differentiated cells. We previously reported that cell toxicity induced by exon 4-expressing APOL1 splice variants coincided with the nuclear translocation of TFEB (44). In contrast, in the current study noncytotoxic APOL1 splice variants did not induce nuclear translocation of
TFEB-GFP, raising the possibility that TFEB may mediate cytotoxicity of exon 4-expressing APOL1 variants. To examine this possibility, we used siRNA, which achieved ∼80% knockdown of endogenous TFEB transcript levels, but we did not find reduced cytotoxicity of the exon 4-containing APOL1 variant A. This indicates that nuclear translocation of TFEB is likely a downstream effect of APOL1 and does not itself mediate APOL1 cytotoxicity. Surprisingly, all tested APOL1 splice variants stimulated accumulation of perinuclear autophagosome aggregates.

Future experiments will have to answer the apparently contradictory observation that nontoxic APOL1 splice variants inhibit autophagy without causing detrimental effects. One possible explanation is that, in contrast to differentiated podocytes, HEK293T cells divide and, thus, do not rely exclusively on autophagy for elimination of waste products. Another possibility is that although macroautophagy is significantly blocked by different APOL1 variants, the effect may be not immediately toxic to the cells cultured in nutrient-rich conditions and analyzed 24 h after transfection of APOL1-expressing vectors. Finally, upregulation of microautophagy (38) or chaperone-mediated autophagy (14) may compensate for impairment of macroautophagy. Indeed, inhibition of macroautophagy was shown to stimulate chaperone-mediated autophagy (19). Thus there is a need to test whether nontoxic APOL1 variants stimulate compensatory pathways, thereby avoiding, at least temporarily, damaging effects to the cells.

In attempting to identify the mechanism of APOL1 cytotoxicity, we have tested the role of caspases using the broad-spectrum caspase inhibitor Z-VAD-FMK and caspase-3 cleavage assays. Results of these experiments did not indicate involvement of caspases in cytotoxicity of exon 4-expressing APOL1 splice variants (not shown). This observation opens the possibility that APOL1 toxicity could be mediated by caspase-independent pathways mediated by dysfunctional mitochondria or endoplasmic reticulum stress. These possibilities are under investigation.

Since increased cytotoxicity of APOL1 splice variants A and B1 is determined by exon 4, which contributes to formation of a signal peptide, we also explored whether secretion and subsequent uptake of these variants correlated with cellular toxicity. Surprisingly, APOL1 splice variants were secreted into the culture medium of transfected cells, including variants that lack exon 4-encoding sequences. We assume that there may be a nonspecific release into the medium due to cell damage; however, this would mainly affect the release of toxic APOL1 variants A and B1. The mechanism of secretion of nontoxic APOL1 variants is unknown, but it may involve alternative molecular determinants or nonclassical protein secretion pathways. In this context, we have shown that APOL1 can be released from HEK293T cells in association with microvesicles/exosomes (44).

Permeabilization of transfected cells with saponin shows that a significant fraction of APOL1 is resistant to saponin extraction, possibly as a result of the association of putative membrane-spanning domains of APOL1 with intracellular membranes, as previously suggested (45). However, it is possible that APOL1 may induce cytotoxicity after being endocytosed. Indeed, in podocytes, secreted APOL1 was shown to contribute to podocyte injury (26). To test this possibility, we used a specific inhibitor of clathrin-mediated endocytosis, dynasore (29). Our results indicate that dynasore did not affect accumulation of APOL1 or improved viability in cells transfected with the cytotoxic variant B1. These findings suggest that, at least in HEK293T cells, APOL1 is not effectively endocytosed through a clathrin-dependent pathway or is rapidly degraded upon internalization through a clathrin-independent pathway. A discrepancy between this and another study (28), which demonstrated APOL1 uptake by clathrin-mediated endocytosis, may be related to the use of different cells or different experimental conditions to inhibit endocytosis.

Thomson et al. (45) demonstrated increased hepatic necrosis in mice transduced with the APOL1 splice variant A carrying G1 and G2 risk alleles, as opposed to the nonrisk (G0) APOL1 variant A. Deletion of the signal peptide reduced, but did not abolish, hepatic necrosis, whereas deletion of the amphipathic helix at the COOH terminus of APOL1 significantly attenuated liver injury. These studies are consistent with our findings showing that APOL1 splice variants lacking exon 4 are less toxic. In the current study we investigated only the G0 version of APOL1 in its different splice variants. Together with prior findings showing cytotoxicity of APOL1 G0, it is possible that all allelic variants of APOL1 are potentially nephrotoxic, with differential thresholds for the G0 vs. G1 and G2 risk alleles that can be further modified by alternative splicing. Therefore, further studies should explore the biological effect of G1 and G2 risk alleles in the context of splice variants that lack exon 4, as well as the effect of various external factors, including viral-mediated responses, on the relative abundance of different splice variants.

In conclusion, we have shown that APOL1 splice variants demonstrate differential toxicity in HEK293T cells, with APOL1 splice variants lacking exon 4-encoding sequences being nontoxic to the cells in the time frame of the experiments. We hypothesize that different expression patterns of the APOL1 splice variants may contribute to kidney disease risk and can partly explain the variable lifetime kidney disease risk between carriers of two APOL1 risk alleles.

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