Gain of glycosylation in integrin α3 causes lung disease and nephrotic syndrome

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Integrins are transmembrane αβ glycoproteins that connect the extracellular matrix to the cytoskeleton. The laminin-binding integrin α3β1 is expressed at high levels in lung epithelium and in kidney podocytes. In podocytes, α3β1 associates with the tetraspanin CD151 to maintain a functional filtration barrier. Here, we report on a patient homozygous for a novel missense mutation in the human ITGA3 gene, causing fatal interstitial lung disease and congenital nephrotic syndrome. The mutation caused an alanine-to-serine substitution in the integrin α3 subunit, thereby introducing an N-glycosylation motif at amino acid position 349. We expressed this mutant form of ITGA3 in murine podocytes and found that hyperglycosylation of the α3 precursor prevented its heterodimerization with β1, whereas CD151 association with the α3 subunit occurred normally. Consequently, the β1 precursor accumulated in the ER, and the mutant α3 precursor was degraded by the ubiquitin-proteasome system. Thus, these findings uncover a gain-of-glycosylation mutation in ITGA3 that prevents the biosynthesis of functional α3β1, causing a fatal multiorgan disorder.

Introduction
The integrin family is comprised of 24 transmembrane heterodimeric αβ glycoproteins that link the extracellular matrix to the cytoskeleton (1). Most integrins connect to actin filaments and reside in cellular adhesion structures designated focal adhesions (FAs), which are highly enriched in tyrosine-phosphorylated proteins and serve as major hubs for signal transduction (2). Integrin-ligand binding can be controlled by conformational changes that tune integrin affinity (3). Furthermore, integrin function depends strongly on trafficking events, which include endocytosis, intracellular sorting and recycling, and delivery of de novo synthesized integrins to the plasma membrane by the biosynthetic route (4, 5). Both α and β subunits are synthesized as precursors. After N-linked glycosylation, folding, and association of the α and β subunits in the ER, the heterodimer is transported to the Golgi network, in which the N-linked high-mannose oligosaccharides are further processed into complex oligosaccharides. Subsequently, several of the α subunit precursors, including α3, are cleaved by proprotein convertases such as furin into a heavy and a light chain, which are held together by a disulphide bond to generate the mature form that is expressed at the plasma membrane (6, 7).

A number of human congenital disorders have been associated with defective integrin-mediated adhesion, including the blistering disorder epidermolysis bullosa (integrin α6β4 in epithelia), the bleeding disorder Glanzmann’s thrombasthenia (integrin αIIbβ3 on platelets), leukocyte adhesion deficiency-1 (β2 integrins on leukocytes), and muscular dystrophy (integrin α7β1 in striated muscle) (8–11). Furthermore, the importance of integrins for cell adhesion in a variety of organ systems has been demonstrated by numerous mouse models targeting either integrin subunits or integrin-associated proteins (12). For example, genetic deletion of the laminin-binding (LN-binding) epithelial integrin α3β1 in mice causes severe lung and kidney defects as well as abnormalities in the epidermis (13–17). In podocytes, which are the epithelial cells of the glomerulus of the kidney, α3β1 maintains cell adhesion under great dynamic stress by binding to LN-511/521 in the glomerular basement membrane (GBM). Integrin-LN binding is reinforced by intracellular connection to the actin cytoskeleton via linker proteins, such as α-actinin or integrin-linked kinase, and by lateral association with the tetraspanin CD151 (18). The pivotal role of this multicomponent adhesion unit in regulating the barrier function of the kidney is illustrated by the severe glomerular defects observed in mice upon (podocyte-specific) deletion of α3, β1, integrin-linked kinase, CD151, or the α5 chain of LN-511/521 (19–25). In addition, mutations in the genes encoding the β2 chain of LN-521, α-actinin-4, and CD151 have been identified in patients suffering from renal disorders, including Pierson syndrome, focal segmental glomerulosclerosis, and hereditary nephritis (26–28).

Here, we report a mutation in the human ITGA3 gene in a patient with interstitial lung disease and congenital nephrotic syndrome. The mutation leads to a gain of glycosylation, which impedes heterodimerization of the α3 precursor with β1 but not its association with CD151. As a consequence, the β1 precursor accumulates in the ER, and the mutant α3 precursor is cleared by ubiquitination and degradation by the proteasome. Thus, we have identified a gain-of-glycosylation mutation in ITGA3 that prevents the biosynthesis of functional α3β1, leading to severe kidney and lung defects.
Results

A point mutation in ITGA3 causes congenital nephrotic syndrome and interstitial lung disease. We identified a patient born prematurely, at a gestational age of 36 weeks, to unaffected nonconsanguineous Dutch parents. She had one unaffected sibling (Figure 1A). At birth, the patient presented with respiratory distress. The electrocardiogram was normal, and no structural heart defects were observed by ultrasound. However, lung biopsies revealed interstitial lung disease. In addition, renal ultrasound revealed unilateral kidney hypoplasia with hydronephrosis on the left side, and the patient was diagnosed with congenital nephrotic syndrome. Growth retardation was prominent, which could in part be attributed to prolonged steroid treatment and intermittent feeding difficulties. The patient died at the age of 7 months due to respiratory insufficiency. We first screened known genes implicated in nephrotic syndrome (NPHS1, NPHS2, PLCE1, WT1, LAMB2, TRPC6, ACTN4, and INF2) and surfactant metabolism dysfunction, which is implicated in perinatal respiratory distress (ABCA3, SFTPB, and SFTPC). No mutations were detected in these genes (K.Y. Renkema, unpublished observations). Subsequently, we conducted genome-wide screening in the patient and both parents for copy number variations (CNVs), using Affymetrix 250k SNP array analysis. The data have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE40405 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40405). Whereas no clinically relevant CNVs were detected, a 19.2-Mb long continuous stretch of homozygosity (36.78–55.97 Mb) was identified on chromosome 17, encompassing 1,261 SNP probes, with SNP A_2105989 and SNP_A_4223473 as bordering SNPs (Figure 1, B and C). The

Figure 1
Identification of homozygosity for an ITGA3 mutation in a patient with interstitial lung disease and nephrotic syndrome. (A) Pedigree of the patient's family. I:1 and I:2, the patient's parents; II:2, the patient; II:1, the patient's sister. (B) Results from the 250k SNP array performed on the patient's DNA. Log2 test-over-reference ratio values (y axis) for SNP loci plotted against the position on chromosome 17 (x axis). Red dots represent the log2 ratio for each SNP locus (top panel). The effective hidden Markov model outcome, with a normal test-over-reference ratio of 0. Blue dots represent the mean log2 ratio of neighboring SNPs on the array, indicating no significant copy number gains or losses (middle panel). Idiogram of chromosome 17. Green marks represent heterozygous SNP calls at particular DNA loci. The red box indicates the homozygous 19.2-Mb region, reflected by a loss of heterozygous loci. The thickness of the blue line represents the likelihood of LOH (bottom panel). (C) Idiogram of chromosome 17: The homozygous region from 17q12 to 17q23.2 is boxed. (D) Sequence analysis of ITGA3 in the patient's parents, the patient, and the patient's sister. The patient is homozygous for a missense mutation (c.1045G>T), resulting in the amino acid substitution p.A349S. Both the parents and the sister are heterozygous for the mutation. (E) Schematic overview of the 26 exons of the ITGA3 gene. The location of the c.1045G>T mutation in exon 7 is indicated by an asterisk. (F) Domain organization of the integrin α3 subunit. The A349S mutation is located between 2 FG-GAP repeats in the extracellular β-propeller domain.
The region contained 566 genes, among which was the ITGA3 gene, which encodes the integrin α3 subunit. The possibility of uniparental disomy of chromosome 17 was excluded by investigating the Mendelian inheritance pattern of 4,854 SNPs that are located on chromosome 17 and which are present on the array (K.Y. Renkema, unpublished observations). We then directly sequenced the ITGA3 coding sequence, which revealed homozygosity for a missense variant c.1045G>T, for which both parents and the healthy sibling were heterozygous (Figure 1D). In contrast, the variant was not observed in 384 chromosomes of ethnically matched (Dutch) individuals. In addition, it has not been annotated in dbSNP, the NHLBI Exome Sequencing Project, or our in-house database of 100 exome-sequencing projects.

The ITGA3 gene extends over 34.5 kb and contains 26 exons. The identified mutation is located in exon 7 (Figure 1E). At the protein level, the mutation causes the substitution of alanine 349 for serine (A349S) in an extracellular domain of α3 designated the β-propeller (Figure 1F). In summary, we have identified a missense mutation in ITGA3 in a patient with interstitial lung disease and congenital nephrotic syndrome.

The A349S mutation prevents integrin α3β1 expression in vivo and causes severe kidney and lung abnormalities. Congenital nephrotic syndrome is characterized by proteinuria, resulting from defects in the filtration barrier of the glomerulus. To characterize the defects that caused the congenital nephrotic syndrome in our patient, we performed histological examination of kidney biopsies. Severe abnormalities were observed, including aberrant glomerular morphology and cystic dilation of tubuli and local protein casts as well as interstitial fibrosis and some inflammatory infiltrate. The GBM was irregularly thickened, and the glomeruli consisted of fewer and wider capillary loops covered by highly variable numbers of podocytes. In addition, mesangiolysis and mesangial hypercellularity, occasional extracapillary proliferation reflecting a loss of capillary integrity, and focal segmental glomerulosclerosis were observed.
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Figure 3
Abnormalities in lung tissue of the patient. (A) H&E staining of a lung section from a healthy individual. Scale bar: 100 μm. (B) H&E staining of a lung section from the patient. Scale bar: 100 μm. (C) Keratin staining showing lining of the thickened alveolar septa with reactive type II pneumocytes in patient lungs. Scale bar: 100 μm. (D) PAS staining demonstrating glycogen deposits in interstitial cells in the lungs of the patient. Scale bar: 25 μm. (E) Lung cryosections of the patient and a control were subjected to indirect immunofluorescence analysis using our homemade antibodies against the cytoplasmic tail of α3 (green) and collagen IV (red) or α6 (green) and nidogen (red). IgG was included as a control for antibody specificity. Nuclei were counterstained with DAPI (blue). Scale bar: 50 μm.

(R. Goldschmeding, unpublished observations; Figure 2A; and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI64100DS1). Ultrastructural analysis revealed partial effacement of foot processes and a disorganized and thickened GBM, displaying a multilayered and pseudo-reticular structure with loose fragments, suggestive of a disturbance of the fusion process (Figure 2B and R. Goldschmeding, unpublished observations).

We then investigated the expression and distribution of individual glomerular components by immunofluorescence analysis of kidney cryosections. In the patient's glomeruli, the integrin α3 subunit could not be detected, neither with antibodies J143 or P1B5, which are directed against the extracellular domain of α3, nor with a homemade antibody that recognizes the cytoplasmic tail (Figure 2C and A. Sonnenberg and R. Goldschmeding, unpublished observations). Some aspecific immunoreactivity was observed in focal patches of cells. These also reacted with control IgG and most likely consist of activated mesangial cells, which are known to express Fc receptors. In addition to the lack of α3, a dramatic decrease in the levels of the β1 subunit was visible, confirming that α3β1 is the main β1 integrin in the glomerulus. Also, the expression of podocin and CD151 seemed reduced in the glomeruli of the patient, whereas expression of the integrin α6 subunit appeared to be slightly increased. Furthermore, there was an increase in the expression and deposition of the GBM proteins LN, nidogen, and collagen IV, confirming the thickening of the GBM observed by electron microscopy (Figure 2C).

Next, we analyzed lung biopsies from the patient. There were also multiple abnormalities in the lung, including widened alveolar septa lined with reactive type II pneumocytes and increased numbers of alveolar macrophages, whereas the alveolar walls in an age-matched healthy individual were lined with flat type I pneumocytes and contained virtually no reactive type II pneumocytes and no alveolar macrophages (Figure 3, A–C). Pronounced pulmonary interstitial glycogenesis was detected by PAS staining (Figure 3D). In addition, we observed an abnormal deposition of surfactant by electron microscopy (R. Goldschmeding, unpublished observations). As in the kidney, α3 could not be detected by immunofluorescence analysis of lung cryosections, whereas the expression of α6 seemed to be increased (Figure 3E). Together, these data show that the A349S mutation leads to the lack of integrin α3β1 expression, causing severe abnormalities in the kidneys and the lungs.

The A349S mutation impairs the expression of mature α3 at the cell surface. To investigate the effect of the A349S mutation in vitro, we isolated glomeruli from Itga3<sup>3/3</sup>Cd151<sup>fl/fl</sup>Trp53<sup>+/-</sup> mice, according to the Dynabead perfusion method, as described previously (29). Outgrowing epithelial cells were sorted for the expression of podocalyxin and the absence of the endothelial cell marker CD31. The floxed Itga3 and Cd151 genes were deleted by adenoviral delivery of Cre-recombinase, and the absence of both proteins was confirmed by flow cytometry (Supplemental Figure 2). We then generated the A349S mutation in cDNA encoding human α3 and stably expressed either wild-type human α3 (α3<sup>WT</sup>) or mutant human α3 (α3<sup>A349S</sup>) in the α3/Cd151-deficient podocytes by retroviral transduction, followed by selection with zeocin. The podocytes were also reconstituted with FLAG-tagged human CD151 to avoid possible differences in binding efficiency of murine CD151 to human α3, because of sequence variability in the α3-binding QRD motif, which is KRD in the mouse (30).

We first determined the cell surface expression of various integrin subunits and CD151 by flow cytometry. In line with the results of immunofluorescence analysis of the patient tissue, α3 was completely absent from the cell surface of podocytes expressing α3<sup>A349S</sup>, and β1 levels were strongly reduced (Figure 4A). In contrast, the cell surface expression of CD151, as well as that of the integrin subunits α2, α5, and α6, was similar in podocytes expressing α3<sup>WT</sup> or α3<sup>A349S</sup>

Next, we analyzed the expression of precursor and mature α3 by Western blotting. The α3 subunit is synthesized as a precursor of approximately 150 kDa. After N-linked glycosylation in the

ER and association with β1, the α3β1 heterodimer is transported to the Golgi. Here, processing of the N-linked oligosaccharides occurs, and the α3 precursor is cleaved into a heavy (~115 kDa) and a light chain (~35 kDa) (6, 7). The β1 subunit is synthesized as a partially glycosylated precursor of approximately 105 kDa and a more heavily glycosylated mature form of approximately 125 kDa that is expressed at the cell surface. Intriguingly, we clearly detected precursor α3A349S, though to a lesser extent than α3WT, suggesting that the mutant is either less expressed or that some degradation occurs. The mature product was nevertheless completely absent, as evidenced by the lack of the α3 light chain (Figure 4B). Consistently, there was a large pool of β1 precursor but a dramatic reduction of mature β1, as compared with the level in podocytes expressing wild-type α3. This observation supports the results obtained by flow cytometry and suggests that the mutant α3 precursor fails to heterodimerize, leading to accumulation of β1 precursor and a strong reduction of mature β1. These data indicate that, whereas the A349S mutation does not impair the expression of precursor α3, it leads to a lack of the mature α3 subunit at the cell surface, probably because heterodimerization with β1 is prevented.

The A349S mutation impairs α3β1 heterodimerization but not the association of precursor α3 with CD151. To determine whether the A349S mutation indeed prevents heterodimerization of the α3 and β1 subunits, we immunoprecipitated α3 from lysates of podocytes expressing α3WT or α3A349S. The α3 subunit was precipitated with either antibodies against its cytoplasmic tail (29A3) or antibodies that recognize its extracellular domain (J143 and P1B5), whereas detection of α3 was performed using our homemade antibodies directed against its cytoplasmic tail. On reducing gels, this antibody recognizes the precursor of α3 as well as the 35-kDa light chain of the mature α3 subunit. In lysates from podocytes expressing α3WT, all antibodies precipitated both mature α3 and the precursor, although the latter was recognized to a lesser extent by J143 and P1B5 than by 29A3. Precursor α3WT appeared as 2 bands, which likely represent the high-mannose form in the ER and the complex form after modification of the N-linked oligosaccharides in the Golgi apparatus. As expected, the β1 subunit was coprecipitated with α3WT. In contrast, neither J143 nor P1B5 precipitated the α3A349S precursor, whereas 29A3 did precipitate precursor α3A349S but not the mature α3 or β1 subunits (Figure 5A). These results suggest that the α3A349S precursor does not associate with β1 and that J143 and P1B5 recognize a complex-dependent epitope on the α3 subunit. We then precipitated the β1 subunit from podocyte lysates, using either the MB1.2 or the 9EG7 antibodies. Intriguingly, 9EG7 almost exclusively precipitated the β1 precursor but little or no mature β1, whereas MB1.2 precipitated both the precursor and mature β1, together with mature α3WT. In contrast, β1, but hardly any mature β1, was precipitated from lysates of podocytes expressing the mutant α3 subunit (Figure 5B). Furthermore, neither of the 2 antibodies against β1 coprecipitated α3A349S. These results confirm that the A349S mutation prevents association of the α3 subunit with β1 in cultured podocytes. To analyze whether heterodimerization was also impaired in the patient, we precipitated α3 with 29A3 from a lysate of patient lung tissue. Consistent with the observations in podocytes, both precursor and mature α3, as well as β1, were coprecipitated with 29A3 from tissue of an unaffected individual, whereas no mature α3 or β1 and only a small amount of precursor α3 was precipitated from patient tissue (Figure 5C). We then investigated the association of α3WT and α3A349S with CD151 in podocytes. For this purpose,
we performed immunoprecipitation experiments using either the 11G5 antibodies against CD151 or M2, which is directed against the FLAG-tag. As a negative control, we included the TS151R antibodies that recognize the QRD sequence in CD151 that interacts with α3 and thus can only bind to CD151 when not in complex with α3β1 (31, 32). Both M2 and 11G5 coprecipitated CD151 with precursor and mature α3WT as well as with β1, suggesting that CD151 can associate both with the α3 precursor and mature α3. In addition, both antibodies clearly coprecipitated CD151 with α3A349S but not with β1, indicating that association of CD151 with the mutant precursor is not impaired and that CD151-α3 association can occur prior to the association of α3 with β1 (Figure 5D).

As expected, the TS151R antibodies only precipitated CD151 and no integrin subunits. These data suggest that CD151 can associate with precursor α3 prior to α3/β1 heterodimerization and that the A349S mutation prevents α3 heterodimerization with β1 but not its association with CD151.

Gain of glycosylation disrupts heterodimerization and cell surface expression. To understand the mechanism by which the A349S mutation impairs α3/β1 heterodimerization, we first determined the position of alanine 349 in the 3-dimensional structure of α3β1 by homology modeling based on the recently resolved crystal structure of α5β1 (33). Alanine 349 is located on a loop opposite to the α/β interface, suggesting that a mutation at this site does not directly impair α3β1 heterodimerization by steric hindrance (Figure 6A).

The A349S substitution introduces a novel N-glycosylation motif in α3 (N-x-A > N-x-S), indicating that it leads to a gain of glycosylation. This was further suggested by the reduced mobility of the α3A349S precursor in gel electrophoresis on low-percentage gels, whereas both the α3WT and the α3A349S precursor migrated with similar mobility after incubation of the precipitated proteins with N-glycanase, an enzyme that cleaves all N-linked high-mannose oligosaccharides (Figure 6B). Alternatively, treatment of cells with tunicamycin, a pharmacological inhibitor of N-linked glycosylation, also abolished the difference in mobility between α3WT and α3A349S precursors, confirming that α3A349S was indeed hyperglycosylated (Figure 6C). However, the removal of all N-linked sugars by tunicamycin did not rescue heterodimerization but instead induced degradation of both the α3 and β1 subunits, reflecting the essential role of N-glycosylation in protein stability. To demonstrate that the addition of a sugar moiety prevents α3/β1 heterodimerization, alanine 349 in α3 was substituted for glycine (α3A349G), which cannot be glycosylated, and the α3A349G mutant was introduced in α3/CD151-deficient podocytes, as described above.
Intriguingly, flow cytometry indicated that α3A349G was expressed at normal levels on the cell surface (Figure 6D). Consistent with this result, immunoprecipitation followed by Western blotting demonstrated that the α3A349G precursor is normally cleaved to generate the mature product and that α3A349G indeed associates with the β1 subunit (Figure 6E). Thus, whereas the A349S substitution has dramatic consequences, substitution of alanine 349 with glycine has no effect. In line with this result, alignment of the amino acid sequence of α3 from different species revealed that a glycine, as well as an arginine or asparagine but not a serine or threonine, at this position occurs naturally in some organisms, further suggesting that sequence variation at this site is not detrimental per se, whereas the introduction of a novel oligosaccharide is (Supplemental Figure 3). Taken together, these data suggest that the A349S mutation leads to a gain of glycosylation, which prevents α3β1 heterodimerization and cell surface expression.

Heterodimerization and cell surface expression of α3A349Gβ1 are not impaired by association with CD151. Complex formation with CD151 requires an extracellular region in the α3 subunit comprising amino acids 570–705, adjacent to the loop containing alanine 349 (32). Because binding of CD151 to the α3A349S precursor is not impaired, we hypothesized that the association of CD151 with α3A349S may affect the orientation of the oligosaccharide in such a way that it induces a conformational change, thereby preventing its heterodimerization with β1. To explore this possibility, we introduced either α3WT or α3A349S, but not CD151, in α3/CD151-deficient podocytes and investigated their expression at the cell surface by flow cytometry. Similarly to that in podocytes that express CD151, α3WT but not α3A349S was expressed at the cell surface in CD151–/– cells (Figure 7A). Accordingly, in lysates of CD151–/– cells reconstituted with α3WT, both the precursor and mature α3 subunits were detected by Western blotting, while in lysates of CD151–/– cells expressing the mutant α3A349S, only the precursor α3 subunit was present (Figure 7B). These results were further confirmed by the lack of expression of mature α3A349S, but not of α3WT, in podocytes that contain CD151, in which the α3-binding glutamine-arginine-aspartic acid (QRD) at amino acid position 194–196 was mutated to isoleucine-asparagine-phenylalanine (INF) (Figure 7B). This demonstrates that the association of CD151 with α3A349S is not the cause of defective α3β1 heterodimerization and cell surface expression.
heterodimerization, cleavage of precursor α3, and cell surface expression and suggests that the introduction of an additional oligosaccharide side chain is by itself sufficient to disturb proper folding of the α3 subunit.

The A349S mutation causes accumulation of the α3 and β1 precursors in the ER, α3 ubiquitination, and posttranslational degradation. We next investigated the subcellular distribution of α3WTβ1 and α3A349Sβ1 in podocytes by confocal microscopy. First, we used the J143 antibodies against the extracellular domain of α3. As expected, α3WT was clearly distributed over the plasma membrane and was enriched laterally at cell-cell contact sites and basally in FAs, as indicated by colocalization with the FA marker phospho-paxillin. In contrast, no α3A349S was detected at the plasma membrane, consistent with the biochemical and flow cytometry data. Furthermore, J143 did not react with precursor α3A349S in intracellular compartments, supporting the idea that this antibody recognizes the α3 subunit only when it is associated with β1 (Figure 8A). In the presence of α3WT, β1 was distributed over the plasma membrane, laterally in cell-cell contacts and basally in FAs, as determined using an antibody directed against phosphorylated paxillin. However, in cells expressing α3A349S, there was hardly any β1 detectable at the plasma membrane, although the FA pool was retained. Instead, a dramatic accumulation was observed in a perinuclear compartment, which was barely visible in cells expressing α3WT (Figure 8B). These observations are consistent with the dramatic reduction in β1 surface expression observed by flow cytometry and the large pool of β1 precursor found in lysates of α3A349S. The perinuclear distribution points, most likely, to the ER-localized excess of β1 that cannot be heterodimerized with α3A349S. We then analyzed α3 localization using our antibody directed against the cytoplasmic domain of α3. Much like the staining pattern obtained with J143, this antibody detected α3WT at the plasma membrane, in cell-cell contacts, and in cell-matrix adhesions. Intriguingly, it also revealed accumulation of α3A349S around the nucleus, where it is colocalized with β1 (Figure 8, C and D). Finally, we examined the distribution of CD151. In cells expressing α3WT, CD151 is colocalized with α3 at the plasma membrane and in adhesions. In cells expressing α3A349S, CD151 was also localized at the plasma membrane, consistent with observations that CD151 can traffic to the cell surface independently of α3 (34). However, a small perinuclear pool colocalized with α3A349S, which probably represents CD151 bound to precursor α3 in the ER (Figure 8E). Cointaining of α3 or β1 with the ER markers protein disulphide isomerase or calnexin confirmed that the perinuclear compartment to which α3A349Sβ1 is localized is indeed the ER (Figure 8F).

Calnexin is a chaperone protein that is involved in ER retention and folding of nascent glycoproteins (35). Both the integrin α and β subunit precursors undergo cycles of folding and unfolding, during which calnexin associates with and dissociates from both to ensure that they reach their proper conformation before being transported to the Golgi (36). To investigate whether α3A349S could still associate with calnexin, we precipitated either β1 or α3 from podocytes expressing α3WT or α3A349S. Both wild-type and mutant α3, as well as β1, coprecipitated from podocyte lysates with calnexin, indicating that the interaction of calnexin was not impaired with either subunit (Supplemental Figure 4). In an attempt to circumvent the quality control mechanism in the ER, we cultured podocytes at 30°C or incubated them with the pharmacological chaperones curcumin or 4-phenylbutyrate. Compounds that can act as chaperones have been used to evade the ER response to misfolded G protein–coupled receptors, including the vasopressin receptor, gonadotropin-releasing hormone receptor, and cystic fibrosis transmembrane conductance regulator (37, 38). Therefore, pharmacological chaperones are suggested as treatment for a variety of diseases due to protein misfolding (39). However, neither strategy induced cell surface expression of α3A349S, whereas the cell surface expression of α3WT was increased (C. Margadant, unpublished observations).

Misfolded proteins are disposed of by the ER-associated degradation pathway, which involves ubiquitination and transport from the ER to the cytosol for proteasomal degradation (40, 41). To inhibit the proteasome, podocytes were incubated for 6 hours with lactacystin (10 μM). Treatment with lactacystin induced a striking accumulation of polyubiquitinated α3A349S precursor, but much less
of α3WT, indicating that a large fraction of the mutant precursor is indeed ubiquitinated and proteasomally degraded (Figure 8G).

Together, these data demonstrate that the misfolded α3A349S precursor is cleared by ubiquitination and subsequent proteasomal degradation, whereas the excess of β1 that is unable to associate with α3A349S is mostly retained in the ER.

**Discussion**

Here, we report on a patient with a novel missense mutation in the ITGA3 gene, for which she is homozygous and which disturbs the biosynthesis of integrin α3β1, resulting in a multiorgan disorder, consisting of interstitial lung disease and congenital nephrotic syndrome. The mutation causes a gain of glycosylation in the α3

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**Figure 8**

The α3A349S mutation causes accumulation of β1 precursor in the ER and ubiquitination and proteasomal degradation of α3. (A) Subcellular localization of α3 was investigated in podocytes expressing either α3WT or α3A349S, using J143 against the extracellular domain of α3 (blue). Filamentous actin (F-actin) was stained with phalloidin (red), and FAs were visualized using an antibody against phosphorylated paxillin [P(Y)pax; green]. (B) Localization of β1 (blue) in podocytes expressing either α3WT or α3A349S. Green indicates phosphorylated paxillin, and red indicates F-actin. (C) Distribution of α3 was investigated using an antibody directed against the cytoplasmic domain (blue), and FAs were detected with an antibody against phosphotyrosines [P(Y); green]. Red indicates F-actin. (D) Colocalization of β1 (green) and α3 (blue). Red indicates F-actin. (E) Distribution of CD151 (green) and α3 (blue). Red indicates F-actin. (A–E) Scale bar: 20 μm. Arrows indicate perinuclear localization of α3A349S and β1 in each panel. (F) Colocalization of α3A349S with protein disulphide isomerase (PDI; top) and β1 with calnexin (bottom). Nuclei were stained with DAPI. Scale bar: 20 μm. (G) Podocytes expressing either α3WT or α3A349S were treated for 6 hours with the proteasomal inhibitor lactacystin (10 μM) and lysed, and ubiquitinated α3/β1 was detected by precipitation of the indicated subunits, followed by Western blotting.
The A349S mutation leads to hyperglycosylation of the \( \alpha3 \) subunit, which prevents heterodimerization with \( \beta1 \) but not the association with CD151. Although the mutant \( \alpha3 \) subunit still interacts with calnexin, its misfolding is terminal, leading to rapid clearance by the ubiquitin/proteasome system while the noncomplexed \( \beta1 \) accumulates in the ER (Figure 9).

The kidney and lung abnormalities observed in the patient are consistent with the defects in kidney and lung morphogenesis observed in \( \alpha3 \)-deficient mice (13). In addition, we found extracapillary proliferation, focal segmental glomerulosclerosis, and interstitial fibrosis in the glomeruli of the patient, which was not observed in \( \alpha3 \)-null mice. The latter observations likely reflect the progressive nature of the glomerular damage, as \( \alpha3 \)-deficient mice die much earlier than \( \alpha3 \)-null mice. In addition to the kidney and lung defects, absence of \( \alpha3 \) in the epidermis causes skin abnormalities, including (micro-)blistering, basement membrane duplication, inflammation, and alopecia (13–17). Although obvious macroscopic skin abnormalities were not observed in the patient described here, we cannot exclude that microblistering or aberrant epidermal basement membrane organization occurred.

During the revision of this article, a report was published describing mutations in \( ITGA3 \). While the kidney and lung defects observed in the patients concerned are largely similar to those described here, an abnormal skin phenotype was also reported (42). It should be noted that the skin defects caused by \( \alpha3 \) deficiency in mice are relatively minor, because adhesion of epidermal keratinocytes to LNs is rescued by other integrins, most notably the hemidesmosome-based integrin \( \alpha6\beta4 \) (17, 43). Thus, redundant mechanisms exist in epidermal keratinocytes to ensure adhesion, which is probably why skin defects are not always detected in the absence of \( \alpha3 \) (44). Similarly, a mutation in \( CD151 \) has been described to cause epidermolysis bullosa, whereas skin defects have not been observed in \( CD151 \)-deficient mice (19, 24, 25, 28). Nevertheless, \( \alpha6 \) integrins cannot compensate for the absence of \( \alpha3\beta1 \) in the kidneys and the lungs, even though \( \alpha6 \) expression seemed to be increased in these organs in the patient described here.

Our immunoprecipitation experiments yield some important conclusions. First, the mutant \( \alpha3 \) subunit does not heterodimerize with \( \beta1 \), as shown by pull-down of either the \( \alpha3 \) subunit with 29A3 or the \( \beta1 \) subunit with MB1.2. Intriguingly, the 9EG7 antibody, which is widely used as a marker for \( \beta1 \) integrins in the active, high-affinity conformation (45), exclusively precipitated precursor \( \beta1 \) but not its mature form. In addition, 9EG7 did not recognize \( \beta1 \) on the cell surface by flow cytometry (C. Margadant, unpublished observations). These observations suggest that there is no \( \beta1 \) in its active conformation at the cell surface, questioning the use of 9EG7 as a bona fide marker for active \( \beta1 \) integrins in general. Second, the J143 and P1B5 antibodies, both directed against the extracellular domain of \( \alpha3 \), apparently recognize a complex-dependent epitope on the \( \alpha3 \) subunit, because they do not precipitate precursor \( \alpha3\Delta349S \), and precipitate much smaller amounts of the \( \alpha3\Delta349S \) precursor than 29A3, but comparable amounts of mature \( \alpha3 \) or \( \beta1 \). The fraction of precursor \( \alpha3 \) that is not recognized by these antibodies is presumably not associated with \( \beta1 \). Third, our data show that CD151 can associate with both the \( \alpha3 \) precursor and mature \( \alpha3 \), which is consistent with previous observations, indicating that CD151-\( \alpha3 \) association occurs early during biosynthesis of \( \alpha3\beta1 \) (34). In addition, it has been shown that CD151 mutants that are not expressed at the cell surface can be coprecipitated with \( \alpha3 \), suggesting that CD151 binds intracellular \( \alpha3 \), of which at least a fraction consists of precursor (34). In fact, the observation that CD151 binds precursor \( \alpha3\Delta349S \), even though the latter does not associate with \( \beta1 \), suggests that CD151-\( \alpha3 \) association occurs prior to \( \alpha/\beta \) heterodimerization.

The A349S mutation leads to hyperglycosylation of the \( \alpha3 \) precursor, which is likely the cause of its disruptive effects, as (a) considerable sequence variability exists at this position among eukaryotes, but a serine or threonine is never encountered, and (b) a mutant carrying a glycine at this position, which cannot be glycosylated, associates normally with \( \beta1 \) and is expressed at the cell surface. As the novel sugar moiety is oriented away from the \( \alpha/\beta \) interface, a direct effect on \( \alpha/\beta \) subunit interaction by steric hindrance seems unlikely. However, it is located between

![Figure 9](http://www.jci.org)
2 FG-GAP repeats, which form β-strands in the β-propeller domain. This region is very important for the proper folding of integrin α subunits, and numerous mutations in the β-propeller of αIIb have been identified that disturb its folding and the heterodimerization with β3 (46). Similar to what we have observed, such mutations lead to proteasomal degradation of the mutant αIIb precursor, whereas the noncomplexed β3 precursor is retained in the ER (47).

The repertoire of gain-of-glycosylation disorders is ever-expanding, and gain-of-glycosylation mutations that disrupt integrin biosynthesis as the cause for a human disorder have previously been identified in the genes encoding integrin subunits β2 (causing leukocyte adhesion deficiency) and β3 (causing Glanzmann’s thrombasthenia) (48–50). We now report a new gain-of-glycosylation mutation that prevents the biosynthesis of integrin α3β1, causing interstitial lung disease and congenital nephrotic syndrome. It will be important to implement ITGA3 gene sequencing in DNA diagnostics for newborns presenting with severe respiratory distress and/or congenital nephrotic syndrome of unknown etiology to facilitate early diagnosis in patients and provide recurrence risk estimation for patients and their relatives.

Methods

Materials. The cDNA encoding CD151 was provided by M. Hemler (Dana-Farber Cancer Institute, Boston, Massachusetts, USA), and the cDNA encoding human α3 has been described previously (51). Mouse mAbs directed against human α3 were J143 (hybridoma from ATCC) and P1B5 (W. Carter, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA). Mouse antibodies against human CD151 were SC11 (F. Berditchevski, University of Birmingham, Birmingham, United Kingdom), TS151R (E. Rubinstein, Hôpital Paul Brousse, Villejuif, France), and 8C3 (K. Sekiguchi, Osaka University, Osaka, Japan). The mouse mAb 29A3 against the cytoplasmic domain of α3A has been described previously (52). Rat mAbs were GoH3 against α6 and MB12 against β1 (B. Chan, University of Ontario, Ontario, Canada). Rabbit polyclonal antibodies were directed against the cytoplasmic domain of α3A (19), the extracellular domain of α6 (A66NT, ref. 53) (A. Cress, University of Arizona, Tucson, Arizona, USA), the cytoplasmic domains of β1A and α2 (G. Tarone, University of Turin, Turin, Italy), calnexin (I. Braakman, University of Utrecht, Utrecht, The Netherlands), collagen IV (E. Engvall, The Burnham Institute, La Jolla, California, USA), LN and nidogen (T. Sasaki, Shriners Hospital for Children Research Institute, Portland, Oregon, USA), and podocin (C. Antignac, Cochin Biomedical Research Institute, Paris, France). The goat antibody used against mouse α3 was AF2787 obtained from R&D Systems. TRITC- and FITC-conjugated secondary antibodies, phallolidin, and DAPI were obtained from Molecular Probes. HRP-conjugated secondary antibodies were obtained from Amersham; curcumin, 4-phenylbutyrate, tunicamycin, and zeocin were obtained from Sigma-Aldrich; and collagen I was obtained from Vitrogen (Nutacon).

Patient material and DNA diagnostics. Peripheral blood samples were obtained from the patient and her parents and sibling after informed consent was given. Genomic DNA was extracted from these samples, and known genes implicated in nephrotic syndrome (NPHS1, WT1, PLCE1, LAMB2, NPHS2, TRPC6, ACTN4, INF2, ABCA3, TTF1, TTF2) and neona- 
tal respiratory distress (ABCA3, SFTPB, and SFTPQ) were screened for variants by Sanger sequencing. Tissue samples used in this study were derived from kidney and lung autopsy or biopsy specimens, obtained with informed parental consent.

SNP array analysis. Genomic DNA was obtained from peripheral blood samples of the patient and her parents. CNV screening by means of microarray analyses was carried out on the Affymetrix GeneChip 250k (NspI) SNP array platform (Affymetrix Inc.), which contains 25-mer oligonucleotides representing a total of 262,264 SNPs. The average resolution of this array platform is 150–200 kb. Hybridizations were performed according to the manufacturer’s protocols. CNVs and long continuous stretch of homozygosity were determined using Copy Number Analyzer for Affymetrix GeneChip mapping software (CNAG version 2.0). The average resolution of this array platform is 150–200 kb.

To investigate uniparental disomy, genotypes were called by Affymetrix Genotype Console Software v2.1.

Mutation analysis of ITGA3. To sequence the complete coding region of ITGA3 in genomic DNA, exon-flanking primers were designed with the Primer3 program (Supplemental Table 1), which is available online (http://frodo.wi.mit.edu/). All coding exons of ITGA3 were amplified by PCR in a total reaction volume of 20 μl, which contained 50 ng DNA, 10 pmol of each primer, 50 mmol of MgCl2, 1 x Taq buffer (Applied Biosystems Inc.), and 1 U Taq polymerase (Applied Biosystems Inc.). PCR products were sequenced in forward and reverse directions by Sanger sequencing on a 3730 DNA analyzer (Applied Biosystems Inc.) with dye-termination chemistry (Big Dye Terminator Cycle Sequencing Kit, version 1.1; Applied Biosystems Inc.). Sequence analysis was performed using Sequencher 4.8 software. The identified gene variant was confirmed by bidirectional sequencing. Segregation analysis was performed by Sanger sequencing. Furthermore, we sequenced the ITGA3 gene in 192 healthy blood donors from The Netherlands. To determine whether the mutation was previously detected in reference populations, we used our in-house database of approximately 100 whole-exome sequencing experiments, dbSNP, and the ExomeVariant Server of the NHLBI Exome Sequencing Project, which is available online (http://evs.gs.washington.edu/EVS/).

Generation of cell lines, cell culture, cloning, and retroviral transduction. Glomeruli were isolated from Itga3–/–Cd151–/–Trp53+/– mice, according to the Dynabead perfusion method as described previously (29), and cultured on 3 μg/ml collagen I at 37°C and 5% CO2 in keratinocyte serum-free medium (K-SFM; Gibco BRL) supplemented with 50 μg/ml bovine pituitary extract, 5 ng/ml EGF, 100 U/ml penicillin, and 100 U/ml streptomycin. Outgrowing epithelial cells were sorted for expression of podocalyxin and absence of CD31. The Itga3 and Cd151 genes were deleted by adenoviral delivery of Cre-recombinase. The A349S and A349G mutations were generated by PCR overlap extension method, using a cDNA encoding human full-length α3A as a template. Wild-type ITGA3 was isolated by digestion with SacI and ligated into pUC18-a3. After digestion with Sphi, ITGA3 was ligated into L2RS-ires-zeo, which was transfected into Phoenix packaging cells using the calcium phosphate method. Virus-containing supernatant was isolated after 48 hours, and stable expression in the α3/CD151-deficient podocytes was achieved by retroviral transduction, followed by selection with zeocin (200 μg/ml). The podocytes were also reconstituted with a cDNA encoding FLAG-tagged human CD151.

Immunoprecipitation and Western blotting. Cell lysis and immunoprecipitation were performed essentially as described previously (17). Whole cell lysates and precipitates were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and analyzed by Western blotting. Bound antibodies were detected using the ECL detection system from GE Healthcare.

Immunohistochemistry, electron microscopy, confocal microscopy, and flow cytometry. Electron microscopy and immunohistochemistry were performed using standard procedures. For indirect immunofluorescence, cryosections from patient biopsies or cover slips with cells were incubated with antibodies as previously described (17). Images were acquired at room
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temperature with a confocal Leica TCS NT or AOBS microscope using x20 dry (numerical aperture 0.7), x40 oil (numerical aperture 1.25), and x63 oil (numerical aperture 1.32) objectives (Leica) and AxioVision 4 software (Carl Zeiss MicroImaging). Pictures were processed using Photoshop 7.0 and ImageJ. Flow cytometry and cell sorting were performed as previously described (17).

Statistics. The 250k Affymetrix microarray data was analyzed using CNAG version 2.0 as previously described (54). CNVs were detected on the normalized log2 ratios using a 5-state hidden Markov model with default settings (–1 μ, –0.3 μ, 0 μ, 0.38 μ, 0.55 μ). These settings were deemed appropriate as the autosomal standard deviation and the sex-mismatch mean ChrX were within the quality control ranges (less than 0.2 and greater than 0.3, respectively). Analysis for loss of heterozygosity (LOH) was also performed using CNAG version 2.0. This algorithm uses a maximum likelihood calculation, with an inference based on the frequencies of heterozygous SNPs to determine significant stretches of LOH. A log-likelihood cut-off value of 15 was used to identify regions of LOH.

Study approval. Written informed consent for SNP array analysis and Sanger sequencing as a part of the diagnostic process (approved by the Medical Ethical Committee of the University Medical Center Utrecht) was obtained from the parents. All studies involving mice were approved by the animal ethics board of The Netherlands Cancer Institute.

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