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Disease-associated single amino acid mutation in the calf-1 domain of integrin α3 leads to defects in its processing and cell surface expression

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ABSTRACT

Integrin α3β1, a receptor for laminins, is involved in the structural and functional organization of epithelial organs, including the lung, kidney, and skin. Recently, a missense mutation that causes substitution of Arg628 with Pro (R628P) in the calf-1 domain of human α3 was shown to be associated with disorders of the lung, kidney, and skin. Here, we found that the R628P mutation leads to aberrations in the posttranslational processing of α3. Specifically, α3 with the R628P mutation showed hardly any cleavage at the calf-2 domain, which usually occurs in the Golgi apparatus during the delivery of de novo-synthesized α3. The mutant α3 retained the ability to associate with integrin β1, but not with the tetraspanin CD151, and the bound β1 was a partially glycosylated immature form, the maturation of which also takes place in the Golgi apparatus. Furthermore, the cell surface expression of the mutant protein was markedly reduced. These results suggest that the R628P mutation leads to a deficit in the transport of α3β1 from the ER to the Golgi apparatus. When Arg628 was mutated to Gln or Glu, instead of Pro, the resulting mutants did not display aberrations in processing or CD151 binding, indicating that the presence of Pro, rather than the absence of Arg, at amino acid residue 628 of α3 is important for the abnormalities in the R628P mutant. In support of this notion, a homology modeling analysis of the calf-1 domain of α3 showed that replacement with Pro, but not with Gln or Glu, caused partial disruption of the β-sheet structures. Furthermore, the ER-associated degradation of the R628P mutant was not enhanced compared with that of the wild-type protein, suggesting that the deficits in the posttranslational processing and cell surface expression of the R628P mutant are independent of the ER-associated degradation, but arise from the defect in its export from the ER. We conclude that the calf-1 domain is required for the transport of α3 from the ER to the Golgi apparatus to maintain the integrity of epithelial tissues, and hence the impairment of the calf-1 domain by the R628P mutation leads to severe diseases of the kidneys, lungs, and skin.

1. Introduction

Integrin α3β1 is a receptor for laminin-511, -521, and -332, the major components of epithelial basement membranes [1]. Integrin α3 is a type I transmembrane protein with a large extracellular region, a single transmembrane helix domain, and a short unstructured cytoplasmic tail [2]. The extracellular region is composed of a seven-bladed β-propeller, a thigh, and two calf domains with flexible linkers, and is posttranslationally cleaved at the calf-2 domain to produce heavy (~115 kDa) and light (~35 kDa) chains that are linked by a disulfide bond [3]. The α3 subunit is expressed in many epithelial organs, including the kidney, lung, and skin, during development, and α3 knockout mice show severe defects in the lungs and kidneys, and aberrations in the epidermis [4,5].

Several mutations of the human *ITGA3* gene have recently been reported to lead to abnormalities of the lungs and kidneys, consistent with the phenotypes of α3 knockout mice [6,7]. Has et al. [6] presented three kinds of mutations: a deletion mutation in exon 8; a point mutation in intron 11 that abolishes the splice acceptor site of exon 12, leading to exon 12 skipping; and a single base substitution in exon 14 that results in a missense mutation, in which Arg628 is changed to Pro. All three mutations produce similar clinical features, such as congenital nephritic syndrome, interstitial

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; mAb, monoclonal antibody; pAb, polyclonal antibody; PBS, phosphate-buffered saline.

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lung disease, and epidermolysis bullosa. Conversely, it remains unclear how the missense mutation in exon 14 causes the disease-associated dysfunction of α3, although the other two mutations induce premature termination. Nicolaou et al. [7] reported that substitution of Ala349 with Ser in α3 results in a gain of glycosylation, causing kidney and lung abnormalities that largely resemble those caused by the above-mentioned three mutations. This mutation not only abolishes the association of α3 with integrin β1, but also causes disorders in its posttranslational processing and cell surface expression, indicating that the biosynthetic processing of α3 from the endoplasmic reticulum (ER) to the plasma membrane is important for the exertion of its biological functions.

In the present study, we found that the missense mutation (replacement of Arg628 with Pro, R628P) in exon 14 of α3 led to a defect in its posttranslational processing, failure of its association with the mature form of integrin β1, absence of its CD151 binding, and a reduction in its cell surface expression. Our results raise the possibility that the R628P mutation induces conformational perturbations of the calf-1 domain of α3 that impair the transport of α3 from the ER to the Golgi apparatus, but do not affect its ER-associated ubiquitin-dependent degradation.

2. Materials and methods

2.1. Cell culture, antibodies and reagents

A549 human lung adenocarcinoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with heat-inactivated 10% (v/v) fetal bovine serum (JRH Biosciences, Lenexa, KS, USA) as described previously [8].

A mouse monoclonal antibody (mAb) against human CD151 (8C3) was produced as described previously [9]. A polyclonal antibody (pAb) against the cytoplasmic tail of human integrin α3A was generated as described previously [10]. An anti-integrin α3 goat pAb and anti-ubiquitin mouse mAb (P4D1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-integrin β1 and anti-calnexin mouse mAbs were from BD Transduction Laboratories (Lexington, KY, USA). Anti-actin rabbit pAb, anti-FLAG mouse mAb (M2), and anti-FLAG mAb (M2)-conjugated agarose were from Sigma. Anti-calnexin rabbit pAb was from Stressgen (Ann Arbor, MI, USA). Peroxidase-conjugated AffiniPure anti-mouse IgG, anti-rabbit IgG, and anti-goat IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Alexa 488-conjugated anti-mouse IgG and Alexa 546-labeled anti-rabbit IgG were from Molecular Probes (Eugene, OR, USA). Lactacystin was obtained from Peptide Institute Inc. (Osaka, Japan).

2.2. Transfection of DNA and siRNA

cDNAs encoding full-length human integrin α3A with a C-terminal FLAG tag and full-length human CD151 were inserted into the pCAGIPuro vector (a kind gift from Dr. Hitoshi Niwa, RIKEN CDB). A cDNA for a siRNA-resistant mutant of CD151 was generated as described previously [11]. The point mutations in α3 were introduced using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The expression vectors were introduced into the cells using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. In transient expression experiments, the cells were subjected to assays at 24–26 h after transfection. Stable transformants expressing wild-type CD151 and its siRNA-resistant mutant were obtained by selection with 2 μg/ml puromycin after transfection.

For RNA interference against CD151, A549 cells were transfected with 50 nM siRNA using Lipofectamine RNAiMAX (Invitrogen) in accordance with the manufacturer's instructions as described previously [11]. At 3 days after transfection, the cells were analyzed by immunoblotting as described below.

2.3. Immunoprecipitation and immunoblotting

Cells were washed with ice-cold phosphate-buffered saline (PBS), and lysed in a lysis buffer containing 1% (w/v) Triton X-100, 5% (v/v) glycerol, 150 mM NaCl, 1 mM EDTA, 20 mM Tris–HCl pH 7.5, and 1 mM phenylmethylsulfonyl fluoride. The lysates were then processed as described previously [11]. For immunoprecipitation, anti-FLAG mAb-conjugated beads (Sigma) were added to the lysates and incubated at 4 °C for 1–3 h. The resulting immune complexes were washed with lysis buffer. The precipitated and lysate proteins were subjected to immunoblotting as described previously [11].

2.4. Cell surface labeling

Cells were washed twice with PBS and surface-labeled with 2 mg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL, USA) in PBS at room temperature for 10 min. After washing with cold DMEM and PBS, the cells were lysed in a buffer containing 1% (w/v) NP-40, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 5% (v/v) glycerol, 150 mM NaCl, 1 mM EDTA, 20 mM Tris–HCl pH 7.5, and 1 mM phenylmethylsulfonyl fluoride. Immunoprecipitation with anti-FLAG mAb-conjugated agarose was performed as described above. The immunoprecipitates were blotted with peroxidase-conjugated streptavidin (Pierce) as described previously [10].

2.5. Immunofluorescence staining

Cells were plated on glass coverslips coated with 10 nM laminin-511, which was purified from conditioned medium of human choriocarcinoma JAR cells as described previously [11]. At 12 h after plating, the cells were transfected with expression vectors for wild-type α3 and its R628P mutant as described above. At 24 h after transfection, the cells were immunostained with anti-FLAG mouse mAb and anti-calnexin rabbit pAb combined with Alexa 488-conjugated anti-mouse IgG and Alexa 546-labeled anti-rabbit IgG secondary antibodies, as described previously [8].

2.6. Homology modeling

Modeling of the calf-1 domain of human integrin α3 was carried out based on the crystal structure of human integrin αV (PDB ID: 3IJE chain A). The amino acid sequences were aligned with those of human integrin α3 (NCBI Reference Sequence: NP_002195) using ClustalW (DNA Data Bank of Japan). A three-dimensional model was generated with SWISS-MODEL. Energy minimization and single amino acid substitutions were then performed using Swiss-PDB Viewer, followed by analysis with UCSF Chimera.

3. Results and discussion

To examine the effects of Arg628 substitution with Pro (R628P) on the expression of integrin α3, wild-type α3 or its R628P mutant tagged with a C-terminal FLAG sequence (α3-FLAG) was transiently expressed in human lung adenocarcinoma A549 cells, followed by immunoprecipitation with an anti-FLAG mAb. Immunoblotting analysis of the precipitates with an anti-FLAG mAb showed that a full-length immature form was detected for both wild-type α3 and its R628P mutant, while the light chain containing the C-terminal FLAG tag was detected for wild-type α3, but not for the R628P mutant (Fig. 1A). Consistent with these results, immunoblotting

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