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Ubc9 interacts with Lu/BCAM adhesion glycoproteins and regulates their stability at the membrane of polarized MDCK cells

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Short title: Ubc9 protein binds to Lu/BCAM glycoproteins

Abbreviations used: gp, glycoprotein; Lu, Lutheran; BCAM, basal cell adhesion molecule; GST, glutathione-S-transferase; kDa, kilodalton; RBC, red blood cell.

Synopsis

Lutheran (Lu) blood group and Basal Cell Adhesion Molecule (BCAM) antigens both reside on two glycoprotein (gp) isoforms, Lu and Lu(v13), that differ by the size of their cytoplasmic tail. They are receptors of laminin-10/11 and are expressed in red blood cells (RBCs), epithelial cells of multiple tissues and vascular endothelial cells. To gain more insights into the biological function of Lu/BCAM gps, we looked for potential partners of their cytoplasmic tail. We isolated Ubc9 (Ubiquitin-conjugating enzyme 9) protein by screening a human kidney library using the yeast two-hybrid system. Lu/Ubc9 interaction was validated by GST pull-down and co-immunoprecipitation experiments. Endogenous Ubc9 formed a complex with endogenous or recombinant Lu gp in A498 and MDCK epithelial cells, respectively. Substitution of lysine 585 by alanine in the Lu gp, abolished *in vitro* and *ex vivo* interaction of Lu gp with Ubc9 protein. Lu K585A mutant transfected in MDCK cells exhibited a normal basolateral membrane expression but was overexpressed at the surface of polarized MDCK cells as compared to wild-type Lu. Pulse-chase experiments showed extended half-life of Lu K585A gp at the plasma membrane, suggesting an impaired endocytosis of this mutant leading to protein accumulation at the membrane. Furthermore, we showed that the ability of MDCK-Lu K585A cells to spread on immobilized laminin was dramatically decreased. Our data supports a physiological role for the direct interaction between Lu gp and Ubc9 protein, and reveals a role for this enzyme in regulating the stability of Lu gp at the cell membrane.

Keywords : Lutheran, BCAM, yeast two-hybrid system, Ubc9

INTRODUCTION

Lu and Lu(v13) are two glycoprotein (gp) isoforms that belong to the immunoglobulin superfamily and which differ only by the length of their cytoplasmic tail (59 and 19 amino acids, respectively) (for reviews, see [1, 2]). These two gps carry both Lutheran (Lu) blood group and Basal Cell Adhesion Molecule (BCAM) antigens [3, 4] and represent adhesion molecules for laminin-10/11 (α5 chain) in normal and in sickle red blood cells (RBCs) [5-7]. In addition to RBCs, Lu/BCAM antigens are predominantly expressed in the endothelium of blood vessel walls, on the surface of a subset of muscle and in the basal membrane of epithelial cells. In contrast to RBCs, Lu/BCAM antigens are highly expressed in epithelial cells [70,000 copies/cell in Caco-2 (colon) or A498 (renal) cells]. Lu gps also exhibit laminin α5 binding properties in endothelial [8] as well as epithelial cells. Indeed, Lu gp is a specific receptor for laminin α5 chain in murine basement membranes [9], and its expression is directly correlated to the expression of laminin α5. In mouse embryos lacking laminin α5, the basal concentration of Lu gp is reduced, whereas the amount of Lu is increased in transgenic mice over-expressing laminin α5 [10]. However, although the interaction between Lu gps and laminin α5 has been clearly demonstrated, the biological function of Lu gps remains unknown.

The 40 C-terminal amino acids specific of the Lu gp cytoplasmic tail carry a dileucine motif involved in the basolateral targeting of this isoform in polarized MDCK epithelial cells [11] and contain phosphorylation sites consistent with a receptor signaling function. We recently demonstrated that PKA-mediated phosphorylation of Lu gp positively regulates its adhesion function to laminin α5 in erythroid cells [12]. Both Lu and Lu(v13) isoforms carry in their common C-terminal cytoplasmic tail the RK573-574 motif necessary for direct interaction with erythroid spectrin [13]. As Lu and Lu(v13) represent quantitatively minor components of the red cell membrane (from 1,500 to 4,000 copies per cell), it is unlikely that these gps play a role in the mechanical properties and the stability of the red blood cell membrane. We have speculated that the interaction with spectrin may be critical for signaling and / or laminin receptor function. In order to identify new cytoplasmic partners of Lu gps that may regulate their expression and / or function, we have screened a human kidney cDNA library using a yeast two-hybrid system and the cytoplasmic domain of the Lu gp isoform as bait. Among the proteins found to potentially interact with the Lu gp, we focused our analysis on Ubc9 (Ubiquitin enzyme conjugating 9), a protein mainly known to conjugate SUMO-1, (a Ubiquitin-like Modifier protein) to proteins. Ubc9 protein represents the only E2-type SUMO-conjugating enzyme described in vertebrates (for reviews, see [14, 15]). We provide evidence for a direct ionic interaction between the lysine 585 of Lu gp and Ubc9 that could play a role in the internalization rate and the turn-over as well as in the adhesion properties of the Lu gp.

MATERIAL AND METHODS

Material

Mouse monoclonal anti-RGS-His6 antibody was purchased from Qiagen (Courtaboeuf, France) and sheep anti-Human Ubc9 (recognizes C-terminal peptide CEYEKRVRAQAKKFAPS) was purchased from A.G.Scientific Inc. (San Diego, CA). Monoclonal antibody (MoAb) anti-Lu^b (clone LM342) was from Dr. R. H. Fraser (Regional Donor Center, Glasgow, United Kingdom), and MoAb anti-Lu, clone F241, was from our institute in collaboration with Dr D. Blanchard (EFS, Nantes) as well as the rabbit polyclonal anti-Lu antibody, 602 (INTS, Les Ulis, Courtaboeuf, France). Mouse MoAb anti-LEX A was from Clontech (Palo Alto, CA) and goat anti-GST from Amersham Biosciences (Buckinghamshire, UK). Mouse monoclonal anti-Glycophorin A (GPA), 3F4, was produced by EFS Loire Atlantique (Nantes, France). Dako was purchased from DakoCytomation (Carpinteria, CA). Monoclonal rat anti-ZO-1 and polyclonal rabbit anti-α catenin antibodies were from Chemicon (Euromedex, France). Rat MoAb anti-E-cadherin antibody was purchased from Interchim (Montluçon, France). Ribonuclease A, purified human laminin-10/11 mixture and 30% bovine serum albumin were purchased from Sigma (St. Louis, MO). Propidium iodide was from Molecular probes - Invitrogen (Leek, The Netherlands). Oligonucleotides used in polymerase chain reaction (PCR) and mutagenesis were purchased from Eurogentec (Seraing, Belgium). Mutagenesis kit (QuickChange XL site-directed mutagenesis) was from Stratagene (La Jolla, CA). Yeast and cell culture media were purchased from Q-Biogene (Montreal, Canada) and Invitrogen, respectively. The pcDNA3 vector was obtained from Invitrogen. The *in vitro* sumoylation assay was performed using the sumoylation control kit distributed by Corgen (Guilford, CT).

Construction of baits

The Lu gp cytoplasmic domain (residues 569 to 628, see Fig.3 for amino acid sequence) was amplified using forward primer 5'GGTGGGAATTCTTTACTGCGTGAGACGCAAAGG3' and reverse primer 5'ACCCGCTCGAGTCAGCACTCGTCTCCGAAG3'. This cDNA *EcoRI* to *XhoI* fragment was fused in frame with the DNA binding domain of LEXA in pLEX12 (a modified pBMT116 plasmid, carrying the tetracycline resistance gene). PLEX-Lu(v13) construct encoding Lu(v13) cytoplasmic domain (residues 569 to 588) was constructed by PCR introducing a stop codon in the pLEX-Lu construct. Oligonucleotides used were: (mutated nucleotides are underlined): sense primer 5'GAAGGGGGCTCCGTAGCCAGGGGAGCCA3' ; antisense primer 5'TGGCTCCCCTGGCTACGGAGCCCCCTTC3'. Mutants of Lu were obtained by *in vitro* mutagenesis. Primers used were as following: for Lu RR572-573AA, sense primer 5'GGAATTCCTACTGCGTGGCAGCAAAAGGGGGCCC3', antisense primer 5'GGGCCCCCTTTTGCTGCCACGCAGTAGGAATTCC3', for Lu K574A : sense primer 5'TACTGCGTGAGACGCGCTGGGGGCCCCTGCTGC3'; antisense primer

5'GCAGCAGGGGCCCCCAGCGCGTCTCACGCAGTA3', for Lu RR582-583AA, sense primer: 5'CCCTGCTGCCGCCAGGCTGCTGAGAAGGGGGCTCC3', antisense primer 5'GGAGCCCCCT TCTCAGCAGCCTGGCGGCAGCAGGG3', for Lu E584A: sense primer 5'CCCTGCTGCCGCCAG AGGAGAGCGAAGGGGGCTCCGCCG3'; antisense primer 5'CGGCGGAGCCCCCTTCGCTCTC CTCTGGCGGCAGCAGGG3'; for Lu K585A : sense primer 5'CTGCCGCCAGCGTCGTGAGGCA GGGGCTCCGCCG3'; antisense primer 5'CGGCGGAGCCCCTGCCTCACGACGCTGGCGGCA G3', and for Lu K585R : sense primer 5'GCCAGCGTCGTGAGAGGGGGGCTCCACCGCC3'; antisense primer 5'GGCGGCGGAGCCCCCCTCTCACGACGCTGGC3'. The inserts were sequenced using ABI-PRISM 310 Genetic Analyzer (Applied Biosystems). Expression of fusion proteins in yeast was verified by Western blot using mouse MoAb anti-LEX A (100 ng/ml).

Yeast Two hybrid Screening and interaction experiments

Two-hybrid screening was performed as described [16], in L40 strain expressing pLEX-Lu cytoplasmic tail transformed with 100 μg of oligo(dT) human kidney cDNA library (Clontech, Ozyme). The library was fused with the activation domain of GAL4 in pACT2. A standard Lithium Chlorure transformation procedure [17] was used and transformation efficiency was estimated on selective medium lacking tryptophane and leucine (DO-WL). His+ clones were selected after 3-5 days of growth at 30°C on selective medium lacking tryptophane, leucine and histidine (DO-WLH). β-galactosidase activity of His+ clones was estimated either on filters, using X-Gal (5-bromo-4chloro-3-Indolyl-β-D-galacto pyranoside), or in liquid assays, using ONPG (O-nitrophenyl β–D-galactopyranoside) on pellets corresponding to 1 ml of culture at OD600 = 0.5. The results are expressed as Miller units = (OD420) x (1000/time) x ($1/OD_{600}$).

pLEX-Lu interacting clones were identified by sequencing of PCR-amplified products obtained from yeast plasmids (forward primer CGATGATGAAGATACCCCACC and reverse primer GAACTTGC GGGGTTTTTCAG). Sequences were submitted to BLAST SEARCH (NCBI).

The Lu(v13) and the Lu mutants baits used in the specificity analysis were pLEX-Lu(v13), pLEX-Lu gp, RR572-573AA, -K574A, -RR582-583AA, -E584A, -K585A and -K585R, constructed as described above. Colonies from co-transformed yeast were picked, applied in duplicate on media DO-WL and DO-WLH and left to grow for 3 days at 30°C. β-galactosidase activity of His+ clones was estimated either on filters or in liquid assays.

GST-Pull Down Assays and Western blots

The Lu gp cytoplasmic domain was expressed in *Escherichia coli* BL21 (Stratagene) as GST fusion protein from pGEX-5X-3 plasmid (Amersham Biosciences) essentially according to Smith *et al.* [18]. After cell lysis by sonication in PBS buffer containing 1% Triton X-100 and protease inhibitor cocktail (Roche) the fusion protein was purified by elution (20 mM glutathion, 50 mM pH: 8 Tris) from

glutathione beads (Amersham Bioscience). Mutants pGEX-5X-3-Lu K574A, K585A, and K585R were constructed by mutagenesis using the Lu plasmid as template. Oligonucleotides used for mutagenesis are as described above for pLEX constructs.

The Ubc9 cDNA (Sequence accession number : NM_020987) was amplified using forward primer 5'TCGCGGATCCTCGGGGATCGCCCTCAG3' and reverse primer 5'ACGCGTCGACCGAT GCCACAAGGTCGC3'. The cDNA *BamHI* to *Sal I* fragment was fused in frame with a His_6-tagged motif in pQE80 plasmid (Qiagen). The His_6-tagged-Ubc9 protein was expressed in *Escherichia coli* BL21. After cell lysis in 1 ml of 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM pH 8 imidazole, 1 mg/ml of lysosyme for 30 min on ice, cells were sonicated on ice and centrifuged at 10000 x *g* for 30 min. The His_6-tagged-Ubc9 protein was purified from the lysate using the Ni-NTA spin column kit (Qiagen).

For *in vitro* interaction, 1 μg (0.05 nmoles) of His6-tagged-Ubc9 protein was incubated with 2 μg (0.05 nmoles) of GST, GST-Lu gp, and the Lu gp mutants Lu K574A, K585A, and K585R for 1 h at 37°C with gentle shaking in 250 mM pH 8 imidazole, 20 mM pH 7.5 Hepes, 75 mM KCL, 1 mM EDTA, 2 mM $MgCl_2$, 2 mM DTT, 0.5 % NP40, and protease inhibitor cocktail. After extensive washings, two successive elutions of proteins bound to glutathione-Sepharose beads were performed (20 mM glutathion, 50 mM pH: 8 Tris).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 15% polyacrylamide gels according to Laemmli [19]. Blots were performed on nitrocellulose membranes [20] and probed with anti-RGS-His6 (1:100000) or anti-Ubc9 (1:3000) MoAb using the enhanced chemiluminescence (ECL) system according to the manufacturer's instructions (Amersham Biosciences).

Cell culture and transfection

Human kidney carcinoma epithelial cells A498 (American Type Culture Collection: HB44) were maintained in Minimal Essential Medium (MEM) Glutamax I supplemented with 10% fetal bovine serum, 100 units/ml antibiotic antimycotic (Invitrogen), 1mM sodium pyruvate, 0.1 mM Non Essential Amino Acids, in a humidified atmosphere at 37°C with 5% CO_2. Madin-Darby canine kidney cells (MDCK, American Type Culture Collection:CCL-34) were maintained in Dubelcco's Modified Eagle's Medium (DMEM) Glutamax I supplemented with 10% fetal bovine serum, 100 units/ml antibiotic antimycotic (Invitrogen), 0.1 mM Non Essential Amino Acids, in a humidified atmosphere at 37°C with 5% CO_2.

Stable MDCK cells expressing wild type and mutated Lu gps generated by *in vitro* mutagenesis as described above for the generation of Y2H baits were obtained as previously described [11]. Briefly, transfected cells were first selected in the presence of 0.6 g/l geniticin and then Lu^b -positive cells were detected by flow cytometry using the anti-Lu^b MoAb LM342 and amplified by a round of selection using magnetic beads coated with anti-mouse IgG (Dynabeads-M-450, Dynal A.S, Oslo, Norway) as recommended by the manufacturer. This procedure generated pools with at least 90% of Lu positive

cells. Cellular clones were also generated by serial limit dilution and clones showing similar Lu antigen membrane expression [specific antibody binding capacity (SABC) units] were selected (538,200 SABC for Lu, 526,270 for K585A and 467,190 for K585R mutants, respectively).

Immunoprecipitation assay

10^7 and 3.10^7 of A498 cells and stably transfected MDCK cells expressing Lu gp wt, Lu K585A, and K585R were lysed for 20 min at room temperature in 50 mM pH 8 Tris HCL, 150 mM NaCl, 1 mM DTT, 1% N–Octyl-β-D-glucopyranoside (Bachem Heidelberg, Germany) with protease inhibitor cocktail. Cell extracts were incubated overnight at +4°C with the anti-Lu^b MoAb or an irrelevant antibody (anti-ZO-1) bound to protein-A-Sepharose (Amersham Bioscience) or incubated only with protein-A-Sepharose as negative control. After extensive washings in lysis buffer, the co-immunoprecipitated proteins were eluted from the protein A sepharose in Laemmli buffer at 100°C for 5min, and analyzed by western blot using a rabbit polyclonal anti-Lu antibody 602 (1:5000) and an anti-Ubc9 MoAb (1:3000).

Confocal fluorescence microscopy

MDCK cells stably transfected with Lu gp wt and the Lu gp mutants K585A and Lu K585R were grown onto 12-mm diameter, 0.4 μM pore Costar Transwell polycarbonate filters (5.10^5 cells/filter). The transepithelial resistance was measured daily using a Millipore Electrical resistance apparatus (Bedford, MA). At days 3, 7, and 11 of culture, polarized cells were fixed 20 min with 4% paraformaldehyde, treated with 50 mM NH_4Cl in phosphate buffer saline (PBS), permeabilized 10 min with 0.5% Triton (PBS) and incubated with F241 anti-Lu MoAb (1:10) in Dako for 1 h at room temperature. Filters were washed with PBS-0.5% BSA and incubated with an Alexa Fluor 488 conjugated anti-mouse antibody (1:200) (Molecular Probes, Invitrogen) for 1 h at room temperature, washed with PBS-0.5% BSA and treated with RNAse before incubation with propidium iodide. Samples were examined by confocal microscopy using a NIKON EC-1 system equipped with 63/1.4 objective.

Membrane steady state expression of Lu gps

Polarized MDCK monolayers expressing Lu or Lu K585A gp mutant were cultured for 7 days onto 12-mm diameter Costar Transwell polycarbonate filters as described above. Cells were incubated at the basolateral side with 0.5 mg/ml Sulfo-NHS-LC-biotin (Pierce) diluted in biotinylation buffer (10 mM Hepes; 150 mM NaCl; 0.2 mM $CaCl_2$; 0.2 mM $MgCl_2$; pH7.5) at 4°C for 30 min. The apical side was incubated with cold PBS. Filters were washed twice with cold PBS, incubated with 10 mM glycine for 10 min at 4°C and washed twice with cold PBS. Filters were excised and cells were lysed in 800 μl of lysing buffer (150mM NaCl, 20mM Tris, pH8.5; 1% Triton X-100) containing protease inhibitor

cocktail (Roche) at 4°C for 45 min. Lysates were centrifuged at 15000 rpm for 20 min and supernatants were incubated with protein-A-Sepharose (Amersham Bioscience) and goat serum at 4°C for 3 hrs. After this preclearing step, Lu gps were immunoprecipitated (IP) by incubating the supernatants with protein-A-Sepharose and anti-Lu mouse MoAb F241 overnight at 4°C. The beads were washed three times with lysis buffer, and Lu gps were eluted with 10% SDS for 5 min at 100°C. Half of the eluted proteins were diluted with Laemmli 3x buffer (total Lu gps) and the other half was diluted in 500 µl of lysis buffer and incubated for 3 hrs with immunopure-immobilized streptavidin beads (Pierce) to isolate membrane Lu gps. Beads were washed three times with lysis buffer and Lu gps were eluted in 20 µl of Laemmli buffer for 5 min at 100°C. Eluates from both steps (total and membrane Lu gps) were run on 8% polyacrylamide gel under reducing conditions and analyzed by immunoblotting using a rabbit polyclonal anti-Lu antibody 602 (1:10000).

Membrane targeting assays of Lu gps

Delivery of newly synthesized Lu and Lu K585A gps to the membrane and their turn-over were determined by performing a pulse-labeled biotin targeting assay. Polarized MDCK monolayers expressing Lu or Lu K585A gps were cultured as described above. Newly synthesized proteins were labeled by adding 150 µCi [^{35}S] methionine / [^{35}S] cysteine (Invitrogen) in the cell culture medium for 20 min incubation at 37°C. Cells were washed with complete medium and incubated in non-radioactive medium for 60, 90, 120, 150 and 180 min at 37°C. After each incubation time, cells were washed twice with cold PBS and incubated at the basolateral side with 0.5 mg/ml Sulfo-NHS-LC-biotin (Pierce). Immunoprecipitation of total Lu gps and isolation of the membrane fraction were performed as described for the steady state experiments. All proteins were analyzed by SDS-PAGE on 8% polyacrylamide gel under reducing conditions followed by western blot probed with rabbit polyclonal anti-Lu antibody 602 (1:10000). Samples of biotinylated Lu proteins were analyzed by autoradiography to quantify the newly delivered membrane proteins as a function of time.

Morphological cell adhesion assay

The plate wells were coated with 2 µg/ml of laminin-10/11 or 1% bovine serum albumin (BSA) overnight at 4°C. Wells were then washed twice with PBS and were subsequently coated with 1% BSA at 37°C for 1 hour before two additional washes. 10^5 MDCK-WT, -Lu or -Lu K585A cells were washed twice with serum free medium and added to the wells. Cells were incubated in serum free medium for three hours at 37°C. After this incubation time, spread and round cells were quantified in four representative areas by microscopy (Leitz, *x100*) using a computerized image analysis system (Biocom VisioL@b 2000). The counted cells were then averaged and presented as percentage of spread cells.

RESULTS

Identification of Ubc9 as Lu cytoplasmic tail partner in yeast two-hybrid screening

Yeast two-hybrid screening was performed using a human kidney cDNA library with the cytoplasmic tail of Lu gp isoform as bait (amino acids 569 to 628). Among 10^6 transformant clones, ten clones representative of five different proteins were retained for further studies among which the Ubiquitin-conjugating enzyme, Ubc9.

Three independent clones were isolated, one clone encoding a full-length Ubc9 and two clones encoding a truncated form of Ubc9 deleted for the first eight amino acids. To control the specificity of Lu/Ubc9 interaction, co-transformation experiments were performed in yeast using pACT2-Ubc9 full-length isolated clone in combination with either empty pLEX or pLEX-Lu. Auxotrophy for histidine and β-galactosidase activity were then assessed. Yeast transformed with pACT2-Ubc9 and pLEX-Lu, but not pLEX were able to grow in the absence of histidine (not shown). β-galactosidase activity was quantified with a liquid assay. Yeast transformed with pACT2-Ubc9 and pLEX-Lu had an activity of 3.15 Miller units while the activity of yeast transformed with pACT2-Ubc9 and pLEX was less than 0.73 Miller units. These results indicated that Ubc9 protein interacts with Lu gp in yeast two-hybrid system.

Lu gp interacts directly *in vitro* with Ubc9 independently of SUMO

To confirm that Ubc9 protein and the cytoplasmic domain of Lu gp interact directly, we performed *in vitro* GST pull-down assays using GST and GST-Lu fusion protein incubated with recombinant His_6-tagged-Ubc9 protein. Coomassie blue staining allowed us to assess that equivalent amounts of GST and GST-Lu were used for each analysis (Fig.1*A left panel*). Western blots of glutathione eluted samples probed with anti-His_6 antibody demonstrated that GST-Lu gp interacted with His_6-tagged-Ubc9 protein in contrast to GST alone (Fig.1*A right panel*). Western blots were also probed with anti-GST antibody to ensure that no degradation of GST proteins occurred during the assay (data not shown). These *in vitro* experiments confirmed the direct interaction between Ubc9 protein and the cytoplasmic domain of Lu gp revealed by the two-hybrid screening.

Ubc9 is known to conjugate SUMO-1 to lysine side-chains in target molecules [14]. As the cytoplasmic domain of Lu, which carries two lysines (amino acids 574 and 585), may be a target for SUMO-1 in addition to Ubc9, we performed an *in vitro* sumoylation assay on GST-Lu using GST-p53 as positive control. As expected, sumoylated form of GST-p53 was detected at 96 kDa (Fig.1*B left panel*). In contrast to GST-p53, we did not observe any sumoylation of GST-Lu, expected at 53 kDa (Fig.1*B right panel*). These results indicated that SUMO could not be conjugated to the cytoplasmic tail of Lu gp in our experimental conditions.

Lu gps interact with Ubc9 in A498 epithelial cells

The association between Lu gp and Ubc9 was further investigated in A498 human epithelial cells by immunoprecipitation assays using monoclonal anti-Lu^b antibody LM342, which recognizes both Lu gp isoforms. As shown in Fig.2, lane L, A498 cells express Lu gp isoforms and Ubc9 endogenously. After immunoprecipitation with the anti-Lu antibody, the presence of Lu gp and Ubc9 were revealed by western blot using the anti-Lu rabbit polyclonal antibody 602 (Fig.2*A*) and the sheep anti-Ubc9 antibody (Fig.2*B*), respectively, indicating that Ubc9 was co-immunoprecipitated with Lu gp. Lu gp and Ubc9 proteins were not detected when an irrelevant antibody (IR) or protein A sepharose (PA) alone were used for immunoprecipitation (Fig.2*B*). These results indicated that the endogenous Lu gp interacts with Ubc9 in A498 epithelial cells.

Mapping of the Ubc9 binding site in the cytoplasmic tail of Lu gp

To determine the minimal domain of the Lu cytoplasmic tail involved in this interaction and to identify the amino acids that interact with Ubc9, we used the cytoplasmic tail of Lu(v13) isoform which is deleted of the 40 C-terminal amino acids of Lu, in yeast two-hybrid experiments (Fig.3*A*). The pLEX-Lu(v13) plasmid (encoding amino acids 569 to 588) was transformed simultaneously with pACT2-Ubc9. Co-transformants were tested for histidine auxotrophy and β-galactosidase activity on filters. We found that pLEX-Lu(v13)/pACT2-Ubc9 co-transformants grew on deficient DO-WLH medium similarly to pLEX-Lu/pACT2-Ubc9 (not shown). Moreover, β-galactosidase activity estimated on filters was identical for Lu and Lu(v13) (Fig.3*A*). Together, these results indicated that the Ubc9 binding site on Lu gps involves amino acids located in the common cytoplasmic sequence of Lu and Lu(v13).

Electrostatic interactions are known to be important for Ubc9 targets in mammalian cells [21]. The common cytoplasmic domain of Lu and Lu(v13) includes two stretches of positively charged amino acids, RRK (amino acids 572-574) and RREK (amino acids 582-585). The RRK motif is entirely conserved between human, bovine, mouse and rat, whereas the RREK motif is partially conserved in these species. In order to map the binding site of Ubc9 in Lu gp cytoplasmic tail, the arginines, the lysines and the glutamic acid of these motifs were first mutated into alanine separately. Interaction with Ubc9 was tested using the yeast two-hybrid system. As shown in Fig.3*A*, the interaction of Lu RR572-573AA, Lu K574A, Lu RR582-583AA and Lu E584A mutants with Ubc9 was conserved, whereas mutation of lysine 585 into alanine completely abolished this interaction. In order to investigate whether the interaction with Ubc9 involves the lysine intrinsically or the positive charge of this amino acid, we mutated lysine 585 into arginine, another positively charged amino acid. Yeast growth on DO-WLH (not shown) and β−galactosidase activity indicated that the Lu K585R mutant interacted with Ubc9 as efficiently as the Lu wild-type construct (Fig.3*A*).

To confirm the involvement of lysine 585 in Ubc9 binding, GST pull-down *in vitro* experiments were performed using purified His^6-tagged-Ubc9 protein and GST-Lu mutants K585A and K585R, and GST-Lu K574A as control. Coomassie blue staining allowed us to assess that equivalent amounts of GST, GST-Lu, GST-Lu K585A and GST-Lu K585R were used for each analysis and that no degradation of GST proteins occurred during the assay (Fig.3*B upper panel)*. As revealed with the anti-Ubc9 antibody, only K585A mutation totally abolished GST-Lu interaction with Ubc9 confirming the loss of interaction observed in yeast (Fig.3*B, lower panel, lane 3*), whereas mutation K585R had no effect on Ubc9 binding to GST-Lu, (Fig.3*B lower panel, lane 4*). Together, results obtained from GST pull-down and yeast two-hybrid experiments pointed out lysine 585 as the critical residue involved in Lu gps binding to Ubc9.

To analyze the effect of these substitutions in the cytoplasmic tail of Lu gp on Ubc9 binding in a cellular context, mutations K585A and K585R were introduced in the full-length Lu cDNA cloned in the pcDNA3 vector. MDCK cells were stably transfected by wild-type or mutant constructs, and immunoprecipitation assays were performed. As control, aliquots of each cellular extract were analyzed by Western blot using anti-Lu (Fig.3*C upper panel, left)* and anti-Ubc9 antibodies (Fig.3*C lower panel, left)* to verify that equivalent amounts of proteins were present in the starting material. As shown in Fig.3*C (lower panel, right),* Ubc9 was co-immunoprecipitated only with Lu and Lu K585R mutant, but not with Lu K585A mutant confirming *ex vivo* that this last mutation leads to a loss of interaction with Ubc9. Equal amounts of Lu gps were revealed in each immunoprecipitated sample, except for non transfected MDCK cells (Fig.3*C upper panel, right)*. It is noteworthy that the interaction site of Ubc9 is conserved between dog and man as endogenous Ubc9 from MDCK cells bound to recombinant human Lu gp.

These results indicated that lysine 585 of the Lu cytoplasmic tail was involved in the interaction with Ubc9 in renal epithelial cells and that the substitution by another positively charged amino acid like arginine maintained Lu/Ubc9 interaction.

MDCK cells normally express Lu K585A mutant at the lateral surface but exhibit abnormal morphology during polarization

When grown on filters, MDCK-Lu and MDCK-Lu K585R pool cells exhibited normal epithelioid shape, from day 3 up to day 11, establishing monolayers of closely apposed cuboid cells with a fluorescent staining of Lu gp restricted to the lateral domain (Fig.4). In contrast, MDCK-Lu K585A cells displayed a persistent different cellular phenotype along the culture on filters. The shape and size of the cells remained irregular, with many non-polygonal elongated cells. In orthogonal projections, cells appeared more flattened and their height was reduced (mean 8 μm versus 20 μm for the MDCK-Lu and MDCK-Lu K585R cells). Cells were normally polarized, as indicated by ZO-1, α-catenin and

E-cadherin staining (data not shown), and expressed Lu K585A protein at the lateral surface indicating that K585A mutation did not disturb the trafficking of Lu gp to the membrane.

To control that these results were not due to different expression levels of recombinant Lu proteins, flow cytometry studies were performed to analyze the surface expression of Lu gps and select equivalent clones (SABC : 538,200 for MDCK-Lu, 526,270 for MDCK-Lu K585A, and 467,190 for MDCK-Lu K585R). Furthermore, immunofluorescence studies of clones expressing different levels of Lu gps showed similar results, indicating that the phenotype of polarized MDCK-Lu K585A cells was associated to the K585A mutation of Lu gp.

Lu K585A is overexpressed at the membrane of polarized MDCK cells

As wild-type and mutated forms of Lu gp were normally polarized in MDCK cells, we investigated the potential role of Ubc9 in regulating the expression and the stability of Lu proteins at the membrane of polarized MDCK cells.

In a first approach, pools of MDCK-Lu and MDCK-Lu K585A cells were analyzed for the steady state expression of Lu gps at the membrane. Sulfo-NHS-LC-biotin was used to label total membrane proteins at the basolateral side of polarized cells. Total Lu gps were immunoprecipitated using anti-Lu monoclonal antibody F241 and the immunoprecipitated proteins were divided into two halves. The first one was directly analyzed by Western-blot to estimate the total amount of Lu gps. The second half was used to estimate the amount of Lu gps present in the cell membrane, after an additional round of capture of biotinylated Lu gps on streptavidin-coupled agarose beads. All the western-blots were probed with the rabbit polyclonal anti-Lu antibody 602. As shown in Fig.5, equivalent amounts of Lu gp were immunoprecipitated from both cell types (IP Total Lu) but the membrane fraction of Lu K585A mutant was clearly higher as compared to Lu wt indicating that Lu K585A is overexpressed at the plasma membrane.

These results were not related to the expression level of recombinant Lu gps since similar comparative data was obtained using two MDCK clones expressing a same level of Lu gps (SABC: 538,200 for MDCK-Lu and 526,270 for MDCK-Lu K585A), with an overexpression of the Lu K585A mutant at the cell membrane after cell polarization (data not shown).

To investigate the overexpression of Lu K585A mutant at the membrane of polarized MDCK cells, the membrane delivery and turn-over of Lu proteins were analyzed using a pulse-chase experiment combined with biotinylation of surface proteins. Pools of polarized MDCK-Lu and MDCK-Lu K585A cells were radio-labeled for 20 min with [^{35}S] methionine / [^{35}S] cysteine then incubated at 37°C for 60, 90, 120, 150 and 180 min in non-radioactive complete medium. Sulfo-NHS-LC-biotin was used to label total membrane proteins at the basolateral side. Total Lu gps were immunoprecipitated using anti-Lu monoclonal antibody F241 and the biotinylated fraction, corresponding to the Lu gp expressed at the cell membrane, was isolated by streptavidin-agarose beads as described for the steady state

analysis. Biotinylated Lu proteins, representing total Lu gp at the membrane, include a radioactive newly synthesized Lu population of variable extent depending on the time of chase. The amounts of radioactive Lu gps at the membrane were determined at 60, 90, 120, 150 and 180 min by SDS-PAGE of biotinylated Lu gps followed by autoradiography (Fig.6*A*). The chase analysis was started at 60 min as our previous studies indicated that the newly-delivered Lu gp at the membrane, after 20 min of radio-labeling, reached a peak between 60 and 90 min of chase [11]. Newly-synthesized Lu and Lu K585A gps were correctly addressed to the basolateral membrane as they were both present at the cell surface after 60 min of chase (see autoradiography on Fig.6*A*). This result indicated that the Lu/Ubc9 interaction was not involved in the membrane targeting of Lu gp, in agreement with the results obtained by confocal analysis (see above). In contrast, there was a significant difference in the turn-over of the wild-type and the mutated Lu gps as Lu K585A mutant showed increased stability and extended half-life at the membrane over time. Indeed, the newly delivered amounts of this mutant at 60 min remained unchanged at the membrane up to 180 min while Lu wild-type proteins decreased during this time.

To ensure that the decrease of radioactive wild-type Lu gp at the membrane was not due to degradation of Lu proteins during the experiment, samples of biotinylated Lu gps from each chase time were analyzed by SDS-PAGE followed by western-blot probed with the rabbit anti-Lu antibody 602. As shown in Fig.6*B*, equivalent amounts of Lu gp were extracted from the membrane at each chase time. Similarly, equivalent quantities of the Lu K585A mutant were present at the membrane through the chase time. Fig.6*B* also shows a clear overexpression of Lu K585A mutant at the membrane when compared to wild-type Lu. This observation strengthened our results obtained in steady state conditions showing that Lu K585A accumulates at the membrane during polarization of MDCK cells.

To quantify the turn-over of both proteins, the intensity of all bands was determined using NIH Image 1.63 and the ratio "radio-labeled biotinylated Lu/total biotinylated Lu" was calculated for each chase time. As shown in Fig.6*C*, radio-labeled Lu gp decreased at the membrane over time with almost half of the proteins being internalized after 120 min as compared to 60 min. Conversely, Lu K585A mutant showed an unusually stable membrane expression. Indeed, newly delivered Lu K585A proteins did not undergo significant internalization up to 300 min of chase (not shown), indicating that the lack of interaction with Ubc9 through lysine 585 could disturb the turn-over of Lu gp at the membrane of polarized MDCK cells.

Clones expressing similar amounts of Lu and Lu K585A gps, as determined by flow cytometry (SABC: 538,200 for MDCK-Lu and 526,270 for MDCK-Lu K585A) and Western blot (Fig.3*C*, upper panel, lanes 2 and 3), gave similar results (data not shown) indicating that the results obtained with the pools were not dependent on the expression level of Lu gps.

Cell spreading on laminin is impaired in MDCK-Lu K585A cells

To test if the K585A mutation has an effect on the function of cell adhesion to laminin, we examined the ability of MDCK-Lu and MDCK-Lu K585A cells to spread on laminin α5 using a morphological adhesion assay. Microwell culture dishes were coated with 2μg of purified laminin-10/11 (containing α5 chain) or BSA as control. As shown in Figure 7, a large number of MDCK-Lu cells spread on the laminin-coated wells after 3 hours of incubation. In contrast, MDCK-WT and MDCK-Lu K585A cells did not spread on laminin and showed a round morphology. Neither the transfected nor the parental cells spread on the wells coated with BSA. This data indicated that the cells expressing the Lu K585A mutant, which did not interact with Ubc9, exhibit impaired morphological adhesion properties to laminin α5 as compared to MDCK-Lu cells.

DISCUSSION

In this study, we have identified the enzyme Ubc9 as a direct cytoplasmic partner of Lu gps, *in vitro* and *ex vivo*. The co-immunoprecipitation of Ubc9 and Lu gp from A498 cells, expressing both proteins endogenously, assesses the physiological meaning of the two-hybrid and GST pull-down interactions.

In addition to epithelial cells, the Lu gp is expressed in the endothelium of blood vessel walls and in RBCs as well as in the erythroid lineage at the end of erythroid maturation [22]. In a first approach to investigate the Lu/Ubc9 interaction in an erythroid context, we have performed a co-immunoprecipitation assay in transfected K562 cells expressing recombinant human Lu. As observed for epithelial cells, Ubc9 was co-immunoprecipitated with Lu gp (unpublished data). These results indicated that the interaction between Ubc9 and Lu gp was not restricted to the epithelial lineage. It could be assumed that this interaction might occur in all cell types where these two proteins are expressed.

Lysine 585 of Lu gp is directly involved in the binding of Ubc9 since mutation K585A abolished the interaction. Conversely, K585R mutation had no deleterious effect on the Lu/Ubc9 interaction, indicating that Ubc9 binds to Lu gp by an electrostatic interaction. Lysine 585 is included in a charged amino acid cluster RREK. We found that lysine 585 was the critical amino acid for Lu gp interaction with Ubc9, since yeast two-hybrid experiments showed that the Lu RR582-583AA and E584A mutants interacted with Ubc9 similarly to the wild-type Lu gp.

Most of the known Ubc9 interacting partners are nuclear proteins or proteins translocated in the nucleus (for review, see [14]). In only rare cases, Ubc9 has been reported to interact with proteins independently of its sumoylation enzymatic activity and to modulate the activity of transcriptional machinery [23, 24]. Our data indicated that Ubc9 interacts with Lu independently of SUMO. First, the sumoylation of molecules by Ubc9 involves a lysine of the targeted protein that cannot be substituted by another charged amino acid like arginine [25-27]. As demonstrated in this report, the substitution of the lysine 585 by an arginine did not impair Lu/Ubc9 interaction and did not alter the morphology of the MDCK epithelial cells. Furthermore, lysine 585 is not included in a SUMO consensus motif (ΨKXE, where Ψ is a hydrophobic amino acid and X any amino acid). Accordingly, *in vitro* sumoylation experiments performed with GST-Lu and GST-P53 used as control, did not reveal a sumoylation of Lu gp.

To investigate the role of Ubc9/Lu interaction in a cellular context, we performed fluorescence imaging using confocal microscopy. These experiments revealed that the Lu K585A mutant was addressed to the plasma membrane and displayed a correct basolateral expression in MDCK polarized cells. In previous studies we demonstrated that the basolateral expression of Lu was associated with the presence of a dileucine motif within the specific 40 amino acids of Lu gp cytoplasmic tail (position

608-609) [11]. Accordingly, we show that Ubc9/Lu interaction is not necessary for correct targeting of the Lu gp to the lateral plasma membrane of epithelial polarized MDCK cells. Thus, Ubc9 is most probably not involved in the trafficking of Lu gp from the Golgi apparatus to the plasma membrane. Assays performed to co-localize Lu and Ubc9 by confocal microscopy were unsuccessful most likely because it is an enzymatic and therefore very rapid interaction. Lu gp is found mainly in plasma membranes whereas Ubc9 has been shown to exhibit mainly nucleocytoplasmic localization [14]. Mouse Ubc9 was found abundantly in microsomal membranes, was less abundant in nuclei, mitochondria and plasma membranes, and was almost absent in the cytosol [28]. In our experiments, Ubc9 was expressed in the cytosol and the nucleus of MDCK cells (not shown). This suggests that the interaction between Lu and Ubc9 could occur in the cytoplasm during the traffic after internalization. Our hypothesis is that a small proportion of Ubc9 could interact rapidly and reversibly with Lu gp to regulate its trafficking during or after internalization.

To gain more insights into the role of the Lu/Ubc9 interaction, we analyzed the turn-over of wild-type and mutant Lu gps in the plasma membrane. We found that Lu K585A mutant had an extended half-life at the membrane as compared to the wild-type, resulting in accumulation of this mutated protein at the cell surface. Mouse Ubc9, mUbc9, has been described to interact directly with glucose transporters GLUT1 and GLUT4 and to modulate their membrane expression levels in opposite directions through a post-transcriptional mechanism [28]. Overexpression of mUbc9 in skeletal muscle cells led to a severe reduction of endogenous GLUT1 in the cells while expression of a dominant negative form of mUbc9 lacking the catalytic site resulted in an overexpression of GLUT1. This could be compared to our reported results as absence of interaction with Ubc9 is related to an accumulation of Lu K585A mutant at the cell surface, suggesting that Ubc9 could play a key role in the turn-over of Lu gp at the membrane through the endocytic pathway.

Lu K585A transfected MDCK cells displayed an abnormal morphology. Cells were certainly polarized, as indicated by ZO-1, α-catenin and E-cadherin fluorescence staining, but they failed to organize as a regular epithelioid shape with high apposed cuboid cells, remaining smaller as compared to MDCK-Lu cells even at day 11 of culture. Non-polarized MDCK-Lu and MDCK-Lu K585A cells exhibited similar amounts of Lu proteins at the membrane as determined by flow cytometry and Western blot (see Fig.3*C*, upper panel, lane 2 and 3). The expression level of Lu K585A mutant at the membrane was dramatically increased during polarization as determined by protein biotinylation and immunoprecipitation (see Fig.5 and 6). We postulate that the turn-over of Lu gp at the membrane is differently regulated in polarized and non-polarized cells and that the atypical morphology of MDCK-Lu K585A cells during polarization could be a consequence of the abnormal high abundance of an adhesion molecule like Lu gp at the cell surface that could disorganize normal cell-cell contact.

Lu/BCAM proteins, as the unique erythroid receptors for laminin α5, play an important role in sickle cell disease where they could contribute to the abnormal adhesiveness of sickle RBCs to the endothelium and the subendothelial matrix. In a recent work, we showed that cell adhesion to laminin

is modulated by phosphorylation of Lu gp [12]. Lu/BCAM proteins have also been recognized as laminin α5 receptors in kidney epithelial cells, in smooth muscle cells and endothelial cell lines [8, 10, 29]. It was previously established that laminin-10 induced spreading of human corneal epithelial cells by interacting with Lu gps [30]. Here, we show that Ubc9 interacts with Lu gp and could influence cell adhesion to laminin. Indeed, morphological adhesion assays to laminin α5 indicate that MDCK-Lu K585A cells in contrast to MDCK-Lu cells, did not spread on laminin. This result suggests that the loss of Lu/Ubc9 interaction has a consequence on cell spreading. Further investigations should document if signaling events induced by Ubc9 binding regulate laminin adhesion properties of Lu gps.

After demonstrating that Lu and Lu(v13) were differentially delivered to the cell membrane of polarized epithelial cells [11], our current study shows for the first time that the turn-over of Lu gp at the cell membrane is modulated by the interaction with Ubc9. Our studies demonstrate that Ubc9 could be a new component of the endocytic machinery of Lu gps and suggest a potential role for Ubc9 in regulating the turn-over of plasma membrane proteins. These findings help to understand the mechanisms that regulate the expression of an adhesion molecule like Lu/BCAM at the cell membrane and could be of interest to further understand and characterize the adhesion function of these molecules in sickle cell disease and during development.

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FIGURE LEGENDS

FIG. 1. *In vitro* interaction between Lu and Ubc9 studied by GST pull-down assays.

A, left panel, Coomassie blue staining showing that equivalent amounts of GST and GST-Lu were used for each analysis and that no degradation of GST proteins occurred during the assay. A, right panel, His6-tagged-Ubc9 protein was incubated with GST or GST-Lu in pull-down assays and the eluted proteins were analyzed by Western blot (WB) using anti-RGS-His6 antibody. Two successive elutions were performed for GST-Lu interaction (lanes 1 and 2). An aliquot of the purified His6-tagged-Ubc9 protein was loaded on the gel as control (Ubc9 lane). B, *In vitro* sumoylation assay with GST-Lu. GST-Lu was incubated with human Aos1/Uba2 (E1), UBC9 (E2) and with or without human SUMO-I. GST-p53 was used as positive control. GST proteins were detected by Western-blot using a sheep anti-GST antibody. Sumoylated form of GST-p53* was detected at 96 kDa. No sumoylated form of GST-Lu was detected at the expected molecular mass (53 kDa). Dimers of GST-Lu were detected at 70 kDa.

FIG. 2. *Ex vivo* interaction of Lu and Ubc9 by co-immunoprecipitation.

10^7 A498 cells were lysed and extracts were subjected to immunoprecipitation (IP) with monoclonal LM342 anti-Lu^b (Lu), irrelevant antibody (IR) and Protein A sepharose (PA). Presence of Lu gps (panel A) and Ubc9 (panel B) were studied by Western blot (WB) in lysates (L) and in IP eluates using a rabbit anti-Lu (602) and a sheep anti-human Ubc9 antibodies, respectively.

FIG. 3. Mapping of the Ubc9 binding site in the cytoplasmic tail of Lu gp.

A, Yeast two-hybrid experiments. Yeast were co-transformed with pACT2-Ubc9 and pLEX (DNA-binding domain alone), pLEX-Lu, -Lu(v13), -Lu RR572-573AA, -Lu K574A, -Lu RR582-583AA, -Lu E584A, -Lu K585A, or -Lu K585R. β-galactosidase activity was determined on filters for each construct. Mutated amino acids are underlined. B, GST pull down assays: upper panel, Coomassie blue staining was used to show that equivalent amounts of GST (1), GST-Lu (2), GST-Lu K585A (3) and GST-Lu K585R (4) were used. B, lower panel, GST pull-down assays. His6-tagged-Ubc9 protein was incubated with GST (1), GST-Lu (2), GST-Lu K585A (3) or GST-Lu K585R (4) in pull-down assays. Proteins were eluted and analyzed by Western blot (WB) using a sheep anti-Ubc9 antibody. An aliquot of the purified His6-tagged-Ubc9 protein was loaded on the gel as control ("control" lane). C, Immunoprecipitation of Lu gp in MDCK cells. Wild-type (1 and 5) and transfected MDCK clones expressing Lu gp (2 and 6) or mutants Lu K585A (3 and 7) or Lu K585R (4 and 8) were lysed and extracts were subjected to immunoprecipitation with monoclonal LM342 anti-Lu^b antibody. Presence of Lu gp (upper panel) and Ubc9 (lower panel) were tested by Western blot (WB) in lysates (1, 2, 3, 4) and in IP eluates (5, 6, 7, 8) using a rabbit polyclonal anti-Lu (602) and a sheep Ubc9 antibodies, respectively.

FIG. 4. Immunofluorescence studies of the Lu mutants in MDCK cells.

Stably transfected MDCK-Lu gp, -Lu K585A and -Lu K585R cells were grown on filters from day 3 (D3) up to day 11 (D11). Confocal imaging shows nuclei (red) and Lu gp (green) fluorescence staining in xy (en face view) and xz (side views) sections. bars = 10μM.

FIG. 5. Expression level of Lu and Lu K585A mutant at the membrane of polarized MDCK cells grown on transwell filters.

Western-blot using rabbit polyclonal anti-Lu antibody (602) showing the total amounts of Lu and Lu K585A gps after immunoprecipitation using mouse anti-Lu antibody (F241) compared to the biotinylated Lu and Lu K585A gps expressed at the membrane,.

FIG. 6. The expression level and the turn-over of Lu and Lu K585A mutant at the membrane of polarized MDCK cells.

MDCK cells expressing Lu and Lu K585A were grown on transwell filters. A, Newly-delivered Lu and Lu K585A gps at the cell surface as determined by autoradiography of biotinylated Lu gps. B, Western-blot showing total amounts of Lu and Lu K585A gps expressed at the membrane at each time of chase, using rabbit polyclonal anti-Lu antibody (602). C, The curves show the ratio of "radio-labeled biotinylated Lu/total biotinylated Lu" at the membrane reflecting the turn-over of newly-delivered Lu and Lu K585A gps at the cell surface as a function of time. The curves represent mean values obtained with cellular pools (Lu: △ n = 2; Lu K585A: □ n = 2) and clones (Lu: ▲ n = 4; Lu K585A: ■ n = 4).

FIG. 7. Morphological cell adhesion assay of MDCK-WT, -Lu and -Lu K585A cells to laminin-10/11.

10^5 MDCK-WT, -Lu or -Lu K585A cells were incubated at 37°C for three hours into wells coated with 2 μg/ml of laminin-10/11 or 1 % BSA. A, Photos showing cell morphology of each cell line. B, The histogram represents the percentage of spread cells after three hours of adhesion to laminin-10/11 or BSA. Each result represents a mean of four experiments.

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Fig.1

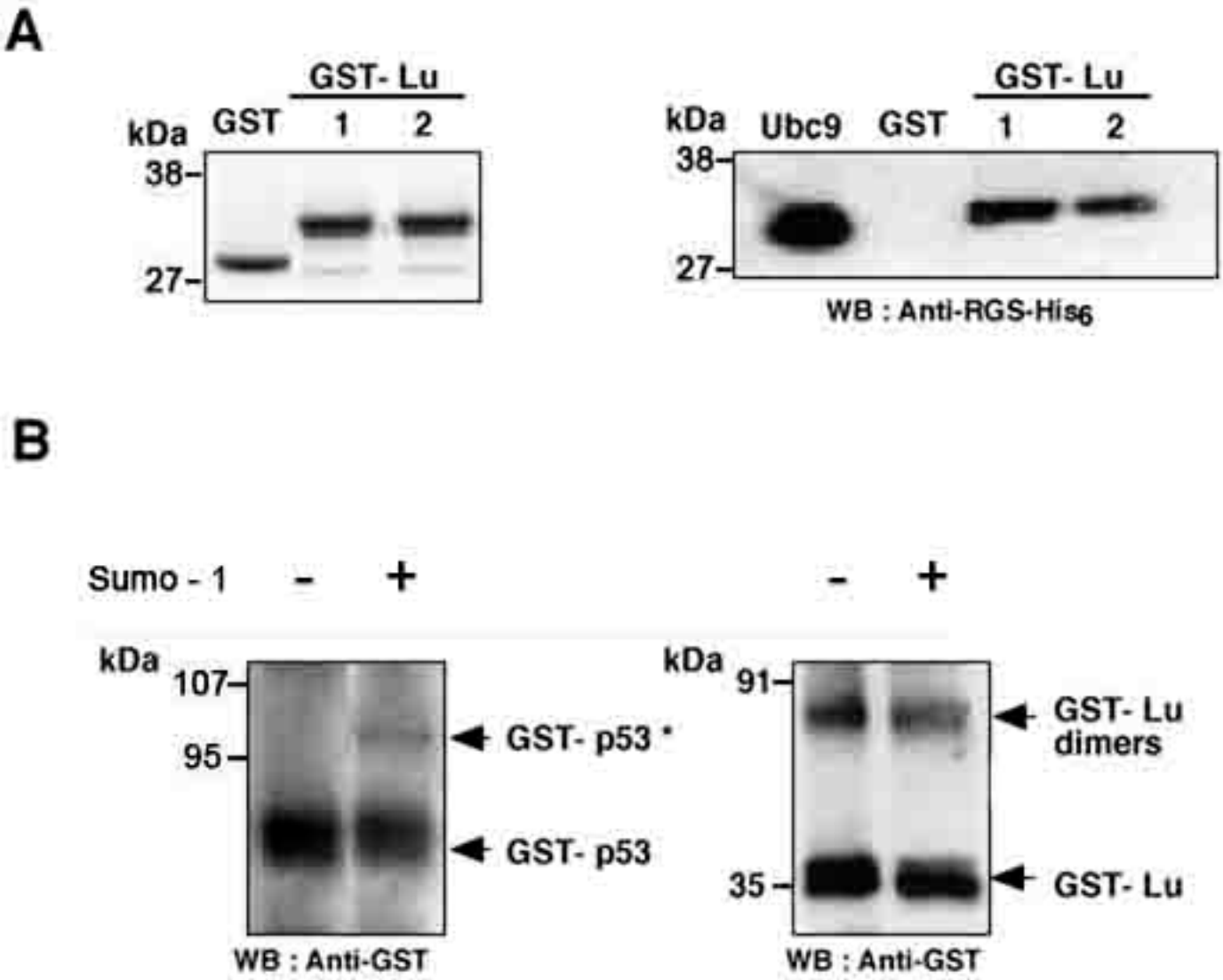

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Fig.2

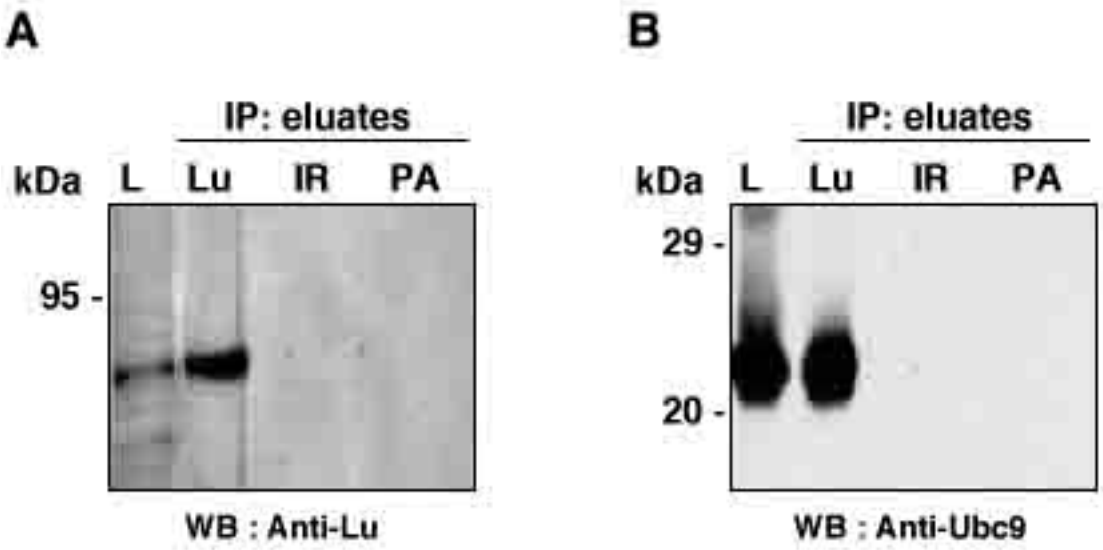

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Fig.3

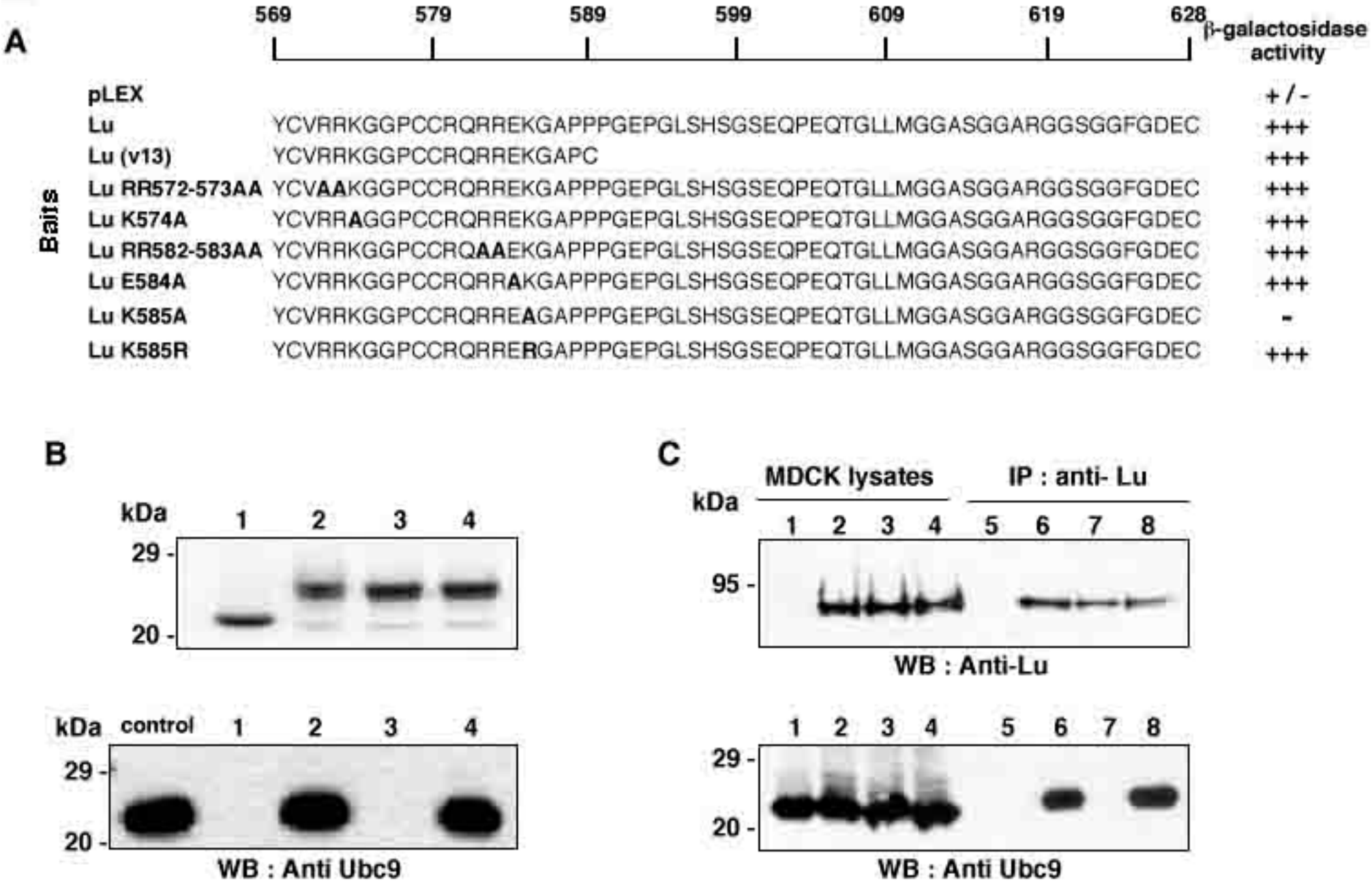

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Fig.4

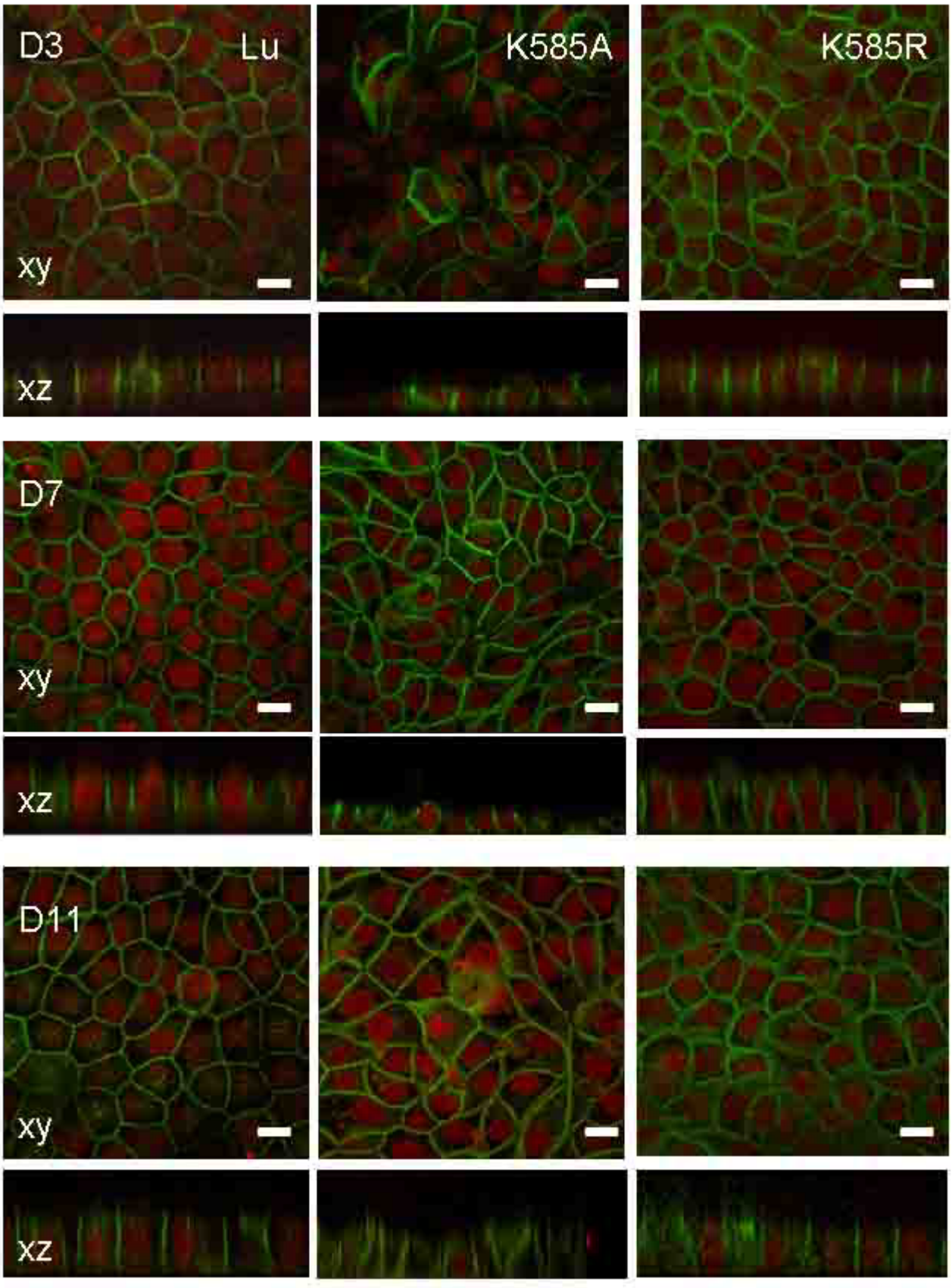

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Fig.5

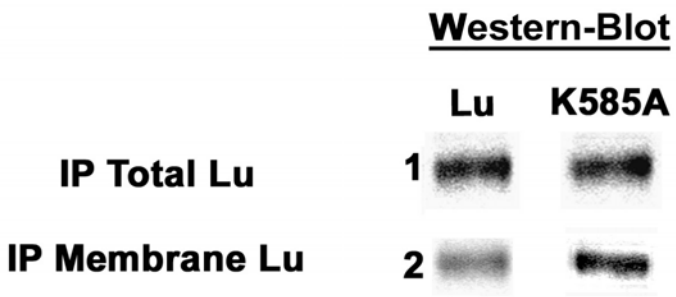

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Fig.6

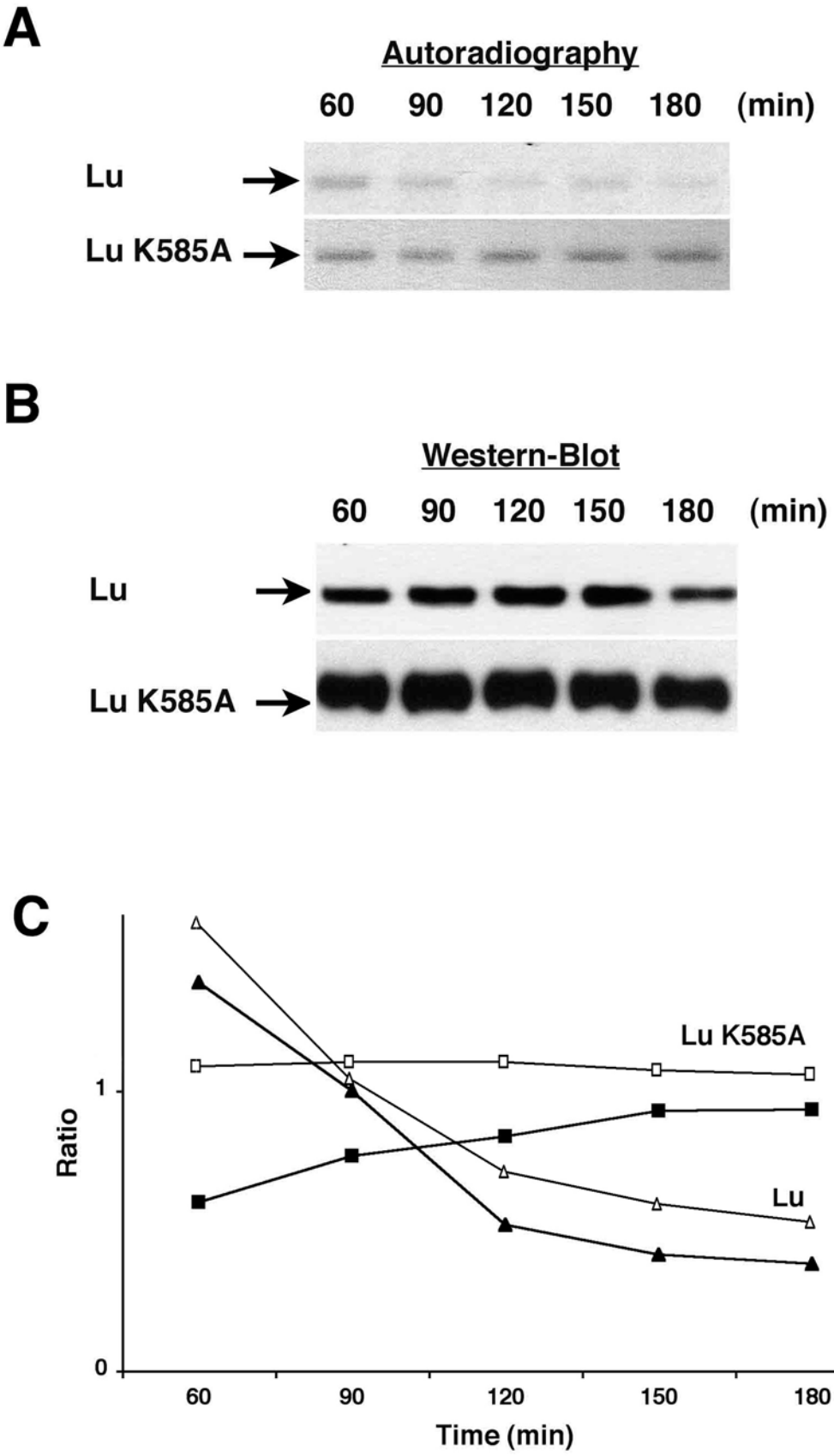

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Fig 7