Myristoylation of human LanC-like Protein 2 (LANCL2) is essential for the interaction with the plasma membrane and the increase in cellular sensitivity to adriamycin

Christine Landlinger, Ulrich Salzer, Rainer Prohaska *

Max F. Perutz Laboratories, Department of Medical Biochemistry, Medical University of Vienna, Dr. Bohr-Gasse 9/3, Vienna A-1030, Austria

Received 9 March 2006; received in revised form 22 June 2006; accepted 24 July 2006
Available online 10 August 2006

Abstract

Human LANCL2, also known as Testis-specific Adriamycin Sensitivity Protein (TASP), is a member of the highly conserved and widely distributed lanthionine synthetase component C-like (LANCL) protein family. Expression studies of tagged LANCL2 revealed the major localization to the plasma membrane, juxta-nuclear vesicles, and the nucleus, in contrast to the homologue LANCL1 that was mainly found in the cytosol and nucleus. We identified the unique N-terminus of LANCL2 to function as the membrane anchor and characterized the relevant N-terminal myristoylation and a basic phosphatidylinositol phosphate-binding site. Interestingly, the non-myristoylated protein was confined to the nucleus indicating that the myristoylation targets LANCL2 to the plasma membrane. Cholesterol depletion by methyl-β-cyclodextrin caused the partial dissociation of overexpressed LANCL2 from the plasma membrane in vitro, whereas in vivo we observed an enhanced cell detachment from the matrix. We found that overexpressed LANCL2 interacts with the cortical actin cytoskeleton and therefore may play a role in cytoskeleton reorganization and in consequence to cell detachment. Moreover, we confirmed previous data that LANCL2 overexpression enhances the cellular sensitivity to the anticancer drug adriamycin and found that this sensitivity is dependent on the myristoylation and membrane association of LANCL2.

© 2006 Elsevier B.V. All rights reserved.

Keywords: Myristoylation; Phosphatidylinositol phosphate; Adriamycin; Actin cytoskeleton; Cell attachment

1. Introduction

The eukaryotic LanC-like (LANCL) protein family is widely distributed in animals, plants, and microorganisms, and shows a clear structural similarity to the prokaryotic lanthionine synthetase component C (LanC) proteins [1]. These LanC proteins are involved in the synthesis of lanthionine-containing antimicrobial peptides known as lantibiotics [2]. Because lantibiotics have not been found in higher eukaryotes, it is likely that LanC-like proteins have different function(s). Very recently, it was suggested that LANCL proteins might catalyze the posttranslational modification of cysteine residues due to their structural similarity to farnesyl transferase [3].

The first member of the family, LANCL1, was isolated from human erythrocyte membranes and the primary structure was determined [4]. Due to seven predicted hydrophobic α-helices in this protein, a function as a G protein coupled receptor was proposed and the gene termed GPR69A. By analogy, the LANCL2 gene was termed GPR69B. Subsequently, detailed immunochemical data revealed that the LANCL1 protein is not an integral but a loosely bound peripheral membrane protein in

Abbreviations: BSA, bovine serum albumin; DOPC, 1,2-Dioleoyl-sn-glycero-3-phosphocholine; DMEM, Dulbecco’s modified Eagle’s medium; GFP, green fluorescent protein; GST, glutathione-S-transferase; HMA, 2-hydroxy myristic acid; LANCL2, lanthionine synthetase component C-like 2; LANCL2-GFP del., N-terminal truncation of C-terminally GFP-tagged LANCL2; LANCL2-GFP mutG1A, C-terminally GFP-tagged LANCL2 with glycine-1 mutated to alanine; mβCD, methyl-β-cyclodextrin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; P-gp, P-glycoprotein; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate(s); PMSF, phenylmethylsulfonyl fluoride; RT, room temperature; TASP, Testis-specific Adriamycin Sensitivity Protein; TBS, Tris-buffered saline; TNET, Tris–NaCl–EDTA–Triton X-100

* Corresponding author. Tel.: +43 1 4277 6160; fax.: +43 1 4277 9616. E-mail address: rainer.prohaska@meduniwien.ac.at (R. Prohaska).

0005-2736/$ - see front matter © 2006 Elsevier B.V. All rights reserved. doi:10.1016/j.bbamem.2006.07.018
erythrocytes [1] and therefore LANCL proteins could not act as G protein coupled receptors. The real function of these proteins is currently unknown.

The human genome contains three LANCL genes, LANCL1 on chromosome 2 [5], LANCL2 on chromosome 7 [6], and LANCL3 on the X chromosome. Northern blot and dot blot analyses of LANCL1 and LANCL2 mRNA in human tissues revealed similar expression patterns with strong expression in various regions of the brain and in testis and weak ubiquitous expression in other tissues [4,7]. LANCL2 mRNA has been isolated and identified in a screen for genes whose down-regulation results in anticancer drug resistance and was therefore also termed Testis-specific Adriamycin Sensitivity Protein (TASP, GenBank accession no. AB035966). Accordingly, Park et al. showed that overexpressed LANCL2 sensitizes cells to the anticancer drug adriamycin due to down-regulation of the multi drug resistance protein, P-glycoprotein (P-gp), concluding a regulatory transcriptional activity for LANCL2 [8].

Amino acid sequence comparison of LANCL2 with the other human LANCL proteins shows a similarity of 75% (LANCL1) and 47% (LANCL3). LANCL2 contains 15 cysteine residues, three of which are conserved in all human LANCL proteins and 8 are conserved between LANCL2 and LANCL1 (Fig. 1).

In this study, we characterized LANCL2 as a plasma membrane associated protein that is N-myristoylated and contains a phosphatidylinositol phosphate (PIP) binding site at the N-terminus. We showed that LANCL2 is associated with the cortical actin cytoskeleton and that overexpression enhances cell detachment upon cholesterol depletion. LANCL2 myristoylation is essential for the membrane association and cellular sensitivity to the anticancer drug adriamycin.

2. Materials and methods

2.1. Drugs and chemicals

[9,10-3H]-Myristic acid was purchased from Moravek Biochemicals Inc. (Brea, CA), D-myo-phosphatidylinositol phosphates: PI, PI(3)P, PI(4)P, PI(5)P were from Echelon (Salt Lake City, UT), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), adriamycin/doxorubicin, 2-hydroxytetradecanoic acid/2-hydroxymyristic acid (HMA), digitonin, filipin III, methyl-β-cyclodextrin (mβCD), cytochalasin B, cytochalasin D, and (3,4-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were from Sigma. Purified LANCL1 was a kind gift of Hemma Bauer.

2.2. Antibodies

Monoclonal antibodies used in this study were anti-GFP (Santa Cruz), anti-GST (Amersham, Uppsala, Sweden), anti-β-actin (Sigma), anti-annexin II (Transduction Laboratories, Lexington, KY) and anti-stomatin GARP-50 as described previously [9]. The polyclonal rabbit anti-GFP antibody was purchased from AbCam (Cambridge, UK) and the polyclonal rabbit anti-LANCL2 antibody 1434 was produced by Sigma-Genosys (Haverhill, Suffolk, UK) against the N-terminus of LANCL2.

![Fig. 1. Multiple alignment of the human LANCL proteins. The amino acid sequences were aligned by CLUSTALW and shaded by BOXSHADE. Identical residues are highlighted by dark grey background, whereas similar residues are shown as light grey boxes. Cysteine residues are boxed in red. The extended N-terminus of LANCL2 and the insertion of 19 residues are marked in yellow. The N-terminal glycine residue, a putative myristoylation site, is marked in blue, and an N-terminal polybasic cluster is boxed. hLANCL1: Swissprot accession number O89112, hLANCL2: Swissprot accession number Q9NS86, hLANCL3: GenBank accession number AAH93667.](image-url)
2.3. Cell line and growth conditions

Human epithelial cells of amniotic origin (UAC) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (PAA Laboratories, Linz, Austria) supplemented with 10% fetal calf serum (PAA) and antibiotics (100 units/ml penicillin and streptomycin) under standard conditions, as described [10].

2.4. Plasmid constructs and mutagenesis

The cDNA of LANCL2 is commercially available as an EST-clone from HGMP Resource Centre (IMAGE number: 50715) that was cloned into the lamin vector at the restriction sites NotI and HindIII. This vector served as template for PCR amplification to subclone LANCL2 into the pEGFP-N3 vector using HindIII and KpnI as restriction sites. The plasmid pEGFP-N3 (Clontech) is a eukaryotic expression vector containing an SV40 origin, a cytomegalovirus promoter, neomycin (G418) selection marker, and codes for the green fluorescent protein (GFP). This vector was used to create the LANCL2-GFP fusion protein with the GFP-tag fused to the C-terminus.

LANCL2 cDNAs coding for the wild-type protein and the N-terminal truncation of 17 amino acids (LANCL2-GFP del.1–17) were generated by PCR and cloned into the unique restriction site HindIII and KpnI of pEGFP-N3. Furthermore, a site-directed mutation of LANCL2, glycine to alanine at position 1 (LANCL2-GFP mutG1A), was generated and cloned into the pEGFP-N3 vector. A Kozak consensus translation initiation site was inserted at the N-terminus of each construct to further increase the translation efficiency in eukaryotic cells. The LANCL1-GFP construct was used for the Bac-to-Bac® system and subsequently cloned into the pEGFP-N3 vector. A Kozak consensus translation initiation site was inserted at the N-terminus of each construct to further increase the translation efficiency in eukaryotic cells. The LANCL1-GFP construct was used for the Bac-to-Bac® system and subsequently cloned into the pEGFP-N3 vector. A Kozak consensus translation initiation site was inserted at the N-terminus of each construct to further increase the translation efficiency in eukaryotic cells. The LANCL1-GFP construct was a kind gift of Hemma Bauer.

2.5. Expression of GST-LANCL2 and purification of the tagged and untagged LANCL2

For expression of GST-LANCL2, the Bac-to-Bac® baculovirus expression system (Invitrogen), LANCL2 was fused to a GST-tag at the N-terminus of the plasmid vector using the unique restriction site HindIII and KpnI of pEGFP-N3. A Kozak consensus translation initiation site was inserted at the N-terminus of each construct to further increase the translation efficiency in eukaryotic cells. The LANCL1-GFP construct was used for the Bac-to-Bac® system and subsequently cloned into the pFastBac1 vector (Invitrogen) according to the manufacturer’s protocol.

For expression of GST-LANCL2, the Bac-to-Bac® system of Invitrogen were used. The cells were infected according to the manufacturer’s protocol. 96 h post-infection, the cells were lysed in phosphate-buffered saline (PBS), pH 7.4, 10 mM DTT, 1 mM EDTA, 0.1% saponin, containing proteinase inhibitors for 20 min at room temperature (RT). Cell debris was removed by centrifugation for 1 h at 65,000×g at 4 °C and the GST-LANCL2 in the supernatant was affinity-purified by GSH Sepharose™ 4B (Amersham). The protein was eluted by TBS, pH 8.0 supplemented with 20 mM glutathione. In order to gain purified LANCL2 without the GST-tag, the GST-affinity-bound GST-LANCL2 was cleaved by thrombin in Tris-buffered saline (TBS), pH 8.0, 5 mM DTT. Subsequently, thrombin was removed by Benzmamidine-Sepharose 6B (Amersham). To concentrate the purified protein, Amicon Ultra 10 MWCO tubes (Millipore, Carrigtwohill, Ireland) were used.

2.6. Stable transfection of UAC cells

C. Landlinger et al. / Biochimica et Biophysica Acta 1758 (2006) 1759–1767

Cells were grown and transfected with LANCL2-GFP or LANCL2-GFP mutG1A by means of Lipofectamine (Invitrogen) according to the manufacturer’s protocol. Within a time period of 2–3 weeks, LANCL2-GFP positive clones formed and were raised. The selecting antibiotic G418 was always supplemented to maintain stable transfection.

2.7. 3H-Myristate cell labeling and immunoprecipitation

UAC cells stably expressing LANCL2-GFP were grown in 100 mm dishes and incubated with 4 ml of DMEM, 2% dialyzed fetal calf serum (PAA) for 1 h. One plate was labeled with [9,10-3H]-myristic acid at 60 μCi/ml by incubation in 5 ml serum-free medium for 4 h at 37 °C. After incubation, the cells were rinsed twice with PBS, lysed in 1 ml of TNET (20 mM Tris–Cl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) containing 1 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, and 5% fetal calf serum, for 10 min at RT. To remove insoluble material, the lysates were centrifuged for 10 min at 15,000×g and 4 °C. The extracts were pre-immunoprecipitated for 1 h with 30 μl of either anti-rabbit or anti-mouse IgG-agarose beads (Sigma) and cleared by centrifugation. For specific precipitation, 30 μl of either anti-rabbit or anti-mouse IgG-agarose were incubated with 1 μg rabbit anti-GFP antibody (In vitro) or 1 μg of the negative control mouse antibody GARP-52, respectively, in TNET for 2 h with gentle agitation. The beads were pelleted, washed 3 times with TNET, mixed with the pre-cleaned lysates supplemented with 1 mg/ml bovine serum albumin (BSA), and incubated for 2 h at 4 °C. The beads were washed 4 times with 4 volumes TNET and eluted with 150 μl of 1.5% Laemmli sample buffer for 1 h at RT. The samples were then separated by SDS-PAGE, gels were treated with Amplify (Amersham) according to the manufacturer’s instructions, and dried. BioMax MS films (Kodak) were exposed for 10 days at –80 °C.

2.8. Membrane preparation and cholesterol depletion

Cells were grown to confluence on a 150 mm dish, subsequently rinsed twice with cold PBS, scraped, and resuspended in 2 ml cold homogenization buffer (10 mM Tris–Hepes, pH 7.4, 30 mM mannitol, 10 mM NaCl, 1 mM pepstatin, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF). The cell suspension was transfected to a chilled Dounce homogenizer and dounced 100 times. The cell lysate was transferred to a Corex tube, centrifuged for 10 min at 4000×g at 4 °C, and the supernatant was removed and ultra-centrifuged for 30 min at 65,000×g at 4 °C. The membrane pellet was then resuspended in 10 mM Tris–Hepes, pH 7.4, 150 mM NaCl, and aliquots were treated with either 15 mM mβ-CD, 10 μg/ml filipin, or 0.01% digitonin, for 15 min at 4 °C. After incubation, the membranes were pelleted by ultracentrifugation for 30 min at 200,000×g and 4 °C.

2.9. Liposome assay

DOPC alone or mixed with PI, P(3)P, P(4)P or P(5)P, respectively, in molar ratios of 25:1 was made up in 95% chloroform/5% methanol and dried under a stream of argon [11]. The lipid film was further dried for 1 h under high vacuum and resuspended in 100 μl hydration buffer (250 mM raffinose, 25 mM HEPES pH 7.4, 1 mM DTT) by vortexing. 300 μl of binding buffer (100 mM sorbitol, 40 mM HEPES pH 7.4, 1 mM DTT, 130 mM KC1, 0.5 mM MgCl2) were added and the sample was sonicated for 1 min at low power in order to obtain small liposome vesicles. The liposomes were pelleted for 10 min at 70,000×g. The pellet was resuspended in binding buffer to a final lipid concentration of 6 mM. 25 μl of liposomes and 0.5 μg of protein (GST-LANCL2) were mixed in binding buffer to give a final volume of 100 μl. The lipid–protein mixture was incubated for 1 h at RT. Then the sample was centrifuged for 10 min at 70,000×g, the supernatant was removed and the pellet washed with 100 μl of binding buffer. The pellet was analyzed by SDS-PAGE and Western blotting.

2.10. SDS-PAGE and Western blot analysis

Proteins were analyzed by 11% SDS-PAGE. For Western blotting, proteins were electrotransferred to nitrocellulose by standard methods along with the pre-stained Protein Molecular Weight Marker (Fermentas, St. Leon-Rot, Germany). Filters were blocked by 3% (v/v) dry milk in 10 mM Tris–Cl, pH 8.0, 150 mM NaCl, 0.1% (v/v) Tween 20 (TBS-Tween) for 1 h, followed by incubation with primary antibody in TBS-Tween for 1 h at RT. The commercial antibodies were used according to the manufacturer’s instructions. Goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Sigma)
were used 1:10,000 in TBS-Tween, and protein bands were detected with SuperSignal™ chemiluminescent substrate (Pierce).

2.11. PIP Strip analyses

The PIP Micro Strips™ (Echelon, Salt Lake City, UT) were blocked with 3% (w/v) fatty acid-free BSA in TBS-Tween by gently agitating for 1 h at RT. Subsequently, the strips were incubated with either 0.5 μg/ml of purified LANCL2, GST-LANCL2, the LANCL2 N-terminal peptide GETMS-KRLKHLGG, the negative control protein LANCL1, or the positive control reagent for PIP Strips, recombinant LL-α domain GST-tagged protein (MultiPIP Grip™, Echelon) for 1–4 h in TBS-Tween. The membranes were rinsed 3 × 5 min in TBS-Tween. The bound proteins were detected by incubation with the primary antibodies (anti-GST for GST-LANCL2 and MultiPIP Grip™, anti-LANCL2 1434 for LANCL2 and the N-terminal peptide, and H60 antibody for LANCL1), the corresponding secondary antibodies, HRP-conjugated anti-mouse and anti-rabbit IgG (Sigma), and the SuperSignal™ chemiluminescent substrate (Pierce).

2.12. Detachment assay

Cells expressing wild-type or mutant LANCL2 were seeded on 6-well plates on the day before the experiment. The nearly confluent cells were treated with 15 mM mβCD for 20 min. After the incubation, the loosely associated cells were 3 times rinsed with a gentle stream of a pipette. The supernatants containing the detached cells were removed and particles of 10–30 μm diameter were counted by means of a cell counter (Z™ Series Coulter Counter®, Beckman). The remaining adherent cells were trypsinized and the cell number of these cells suspensions was also measured by the Coulter counter. The percentage of detached cells after treatment with mβCD was calculated.

2.13. Cell proliferation assay

Different transfected clones were plated at a density of 8000 cells/well in a 96-well plate and cultured under standard conditions. After incubation overnight, adriamycin was added to the cultures to give final concentrations of 0, 250, 1000, 2000, 4000, and 8000 nM. Cells were grown in the presence of the drug for 3 days, and subsequently an MTT assay was performed. The absorbance was determined at 570 nm using a microplate reader (Labsystems Multiskan RC, Life Science, UK).

2.14. Confocal fluorescence microscopy

Stably or transiently transfected UAC cells were grown on cover slips under standard conditions. For some experiments, cells were treated with cytochalasin B or cytochalasin D, at a concentration of 0.5 μg/ml for 2 h or with 10 μM HMA for 24 h. The cells were fixed in freshly prepared 3% (w/v) paraformaldehyde in PBS. To stain nucleoli, the monoclonal antibody 3G4B2 was used and detected by Alexa Fluor® 594 goat anti-mouse IgG antibody. Actin filaments were stained with rhodamine–phalloidin according to the manufacturer’s protocol (Molecular Probes). The cover slips were mounted with Vectashield (Vector Laboratories, Burlingame, CA). Images were collected using a confocal microscope (TCSNT, Leica) and the corresponding software.

3. Results

3.1. LANCL2 is a membrane associated protein

To study the subcellular localization of LANCL2 compared to LANCL1, we transfected the human cell line UAC with LANCL1-GFP and LANCL2-GFP, respectively, and visualized the expressed proteins by fluorescence microscopy. Immunofluorescence data of the endogenous LANCL2 protein were not reliable because of the low expression level of this protein and the low specificity of the LANCL2 antibody in immunocytochemical applications. LANCL2-GFP strongly localized to the plasma membrane and juxta-nuclear vesicles that partially colocalized with the late endosomal markers Lamp1 and Lysotracker (data not shown), whereas LANCL1-GFP was absent from the plasma membrane and exclusively found in the cytosol and nucleus (Fig. 2). The expression of the respective myc-tagged proteins showed the same localization (data not shown). The different localization raised the question whether the membrane association of LANCL2 is caused by the specific N-terminal region, containing an N-terminal glycine residue and a polybasic stretch, or palmitoylation of one or more cysteine residues not conserved in LANCL1 (Fig. 1). We constructed an N-terminal deletion starting at position 18 (LANCL2-GFP del.1–17) with a conserved methionine residue equivalent to the start methionine of LANCL1 (Fig. 1). The stable expression of this construct was analyzed by fluorescence microscopy and revealed the expression in the cytosol and nucleus, but not at the plasma membrane or the juxta-nuclear pool (data not shown), indicating that the N-terminus is crucial for the membrane-association.

3.2. N-Myristoylation of LANCL2 is crucial for the membrane association

To study the relevance of the N-terminal glycine residue as a putative myristoylation site, we mutated this glycine to an alanine. After the stable transfection of UAC cells with this LANCL2-GFP mutG1A construct we observed the localization by confocal fluorescence microscopy. LANCL2-GFP mutG1A was not associated with the plasma membrane but localized almost exclusively to the nucleus, particularly to the nucleoli (Fig. 3A). The same result was obtained when we treated cells stably expressing wild-type LANCL2-GFP with 2-hydroxy-myristic acid (HMA), an inhibitor of N-myristoyltransferases [12,13] (Fig. 3B). These data strongly suggest that the myristoylation of LANCL2 is essential for membrane-association. To confirm the incorporation of myristate into the protein, stably expressing LANCL2-GFP cells were metabolically labeled with [12,13](Fig. 3 B). These data strongly suggest that the myristoylation of LANCL2 is essential for membrane-association.

LANCL1-GFP

LANCL2-GFP

Fig. 2. Different localization of LANCL1-GFP and LANCL2-GFP. UAC cells were (A) transiently transfected with LANCL1-GFP or (B) stably transfected with LANCL2-GFP. The cells were fixed and analyzed by confocal fluorescence microscopy. Bars=10 μm (A, B).
labeled with \(^{3}\)H-myristic acid and subsequently LANCL2-GFP was immunoprecipitated with an anti-GFP antibody. As expected, the autoradiogram showed a band corresponding to LANCL2-GFP labeled with \(^{3}\)H-myristic acid (Fig. 3C). The immunoprecipitation was specific because no signal was found in the negative control with an irrelevant antibody (Fig. 3C).

3.3. PIP-binding as an additional membrane attachment of LANCL2

Due to the limited hydrophobicity of the myristoyl anchor, additional membrane attachment factors may be required for enhanced membrane targeting [14]. Because the LANCL2 sequence does not contain a cysteine residue nearby the N-terminal glycine for palmitoylation (Fig. 1), we focused on the N-terminal polybasic region KRLKLH (Fig. 1, boxed residues 6–11) of LANCL2 that could possibly interact with negatively charged phospholipids, particularly PIPs. Therefore, we incubated PIP Strips™ (Echelon) with the purified LANCL2, the GST-LANCL2 fusion protein, the LANCL2 N-terminal peptide GETMSKRKLHLLGG (residues 1–14), and a positive control reagent for PIP Strips, the recombinant LL5-\(\alpha\) domain GST-tagged protein, respectively. The GST-tagged proteins were detected with the anti-GST antibody, whereas LANCL2 and the GST-tagged LANCL2 showed a specific interaction with PI monophosphates, and a faint reaction with other PIPs (Fig. 4A). The N-terminal peptide alone was sufficient to recognize PIPs, particularly PI(5)P and to a lesser extent the other PIPs and phosphatidic acid (PA) (Fig. 4A). The LL5-\(\alpha\) domain showed a strong interaction with all PIPs, whereas the homologous protein LANCL1 that does not contain labeled with \(^{3}\)H-myristic acid and subsequently LANCL2-GFP was immunoprecipitated with an anti-GFP antibody. As expected, the autoradiogram showed a band corresponding to LANCL2-GFP labeled with \(^{3}\)H-myristic acid (Fig. 3C). The immunoprecipitation was specific because no signal was found in the negative control with an irrelevant antibody (Fig. 3C).

3.3. PIP-binding as an additional membrane attachment of LANCL2

Due to the limited hydrophobicity of the myristoyl anchor, additional membrane attachment factors may be required for enhanced membrane targeting [14]. Because the LANCL2 sequence does not contain a cysteine residue nearby the N-terminal glycine for palmitoylation (Fig. 1), we focused on the N-terminal polybasic region KRLKLH (Fig. 1, boxed residues 6–11) of LANCL2 that could possibly interact with negatively charged phospholipids, particularly PIPs. Therefore, we incubated PIP Strips™ (Echelon) with the purified LANCL2, the GST-LANCL2 fusion protein, the LANCL2 N-terminal peptide GETMSKRKLHLLGG (residues 1–14), and a positive control reagent for PIP Strips, the recombinant LL5-\(\alpha\) domain GST-tagged protein, respectively. The GST-tagged proteins were detected with the anti-GST antibody, whereas LANCL2 and the N-terminal peptide were identified

by the anti-LANCL2 antibody that recognizes the N-terminus. LANCL2 and the GST-tagged LANCL2 showed a specific interaction with PI monophosphates, and a faint reaction with other PIPs (Fig. 4A). The N-terminal peptide alone was sufficient to recognize PIPs, particularly PI(5)P and to a lesser extent the other PIPs and phosphatidic acid (PA) (Fig. 4A). The LL5-\(\alpha\) domain showed a strong interaction with all PIPs, whereas the homologous protein LANCL1 that does not contain
the N-terminal polybasic region did not interact with PIPs at all (Fig. 4A). In order to study the association of LANCL2 with PI monophosphates by another method, we established a liposome binding assay. DOPC liposomes enriched with different PI monophosphates were incubated with purified GST-LANCL2, washed and pelleted, and the associated protein was detected by immunoblotting. A strong association of LANCL2 with PI(3)P and PI(5)P liposomes was observed, whereas the binding to PI (4)P liposomes was not significantly higher than to pure DOPC liposomes (Fig. 4B). As a control, GST-LANCL2 was incubated without liposomes to check whether protein precipitation occurs during the procedure. From these data we conclude that the non-homologous N-terminal basic region of LANCL2 contains a PIP-binding domain that is probably enhancing the membrane binding of LANCL2.

3.4. Cholesterol depletion of LANCL2 expressing cells causes membrane dissociation of LANCL2 in vitro and cell detachment in vivo

It has previously been shown that the PIP2-binding, peripheral membrane protein annexin II is specifically released by sequestration of membrane cholesterol [15,16].

To address the question whether the membrane cholesterol level also affects the membrane association of LANCL2, we performed cholesterol extraction experiments on isolated membranes. We prepared membranes of LANCL2 expressing cells and incubated these with the cholesterol sequestrating agents, digitonin, filipin, and mβCD. Digitonin had the greatest effect and released about 50% of the membrane-bound LANCL2-GFP and about 50% of the membrane-associated actin. As described previously [16], annexin II was completely released from the membrane (Fig. 5A) whereas the integral membrane protein stomatin remained on the membrane indicating that the membranes were not solubilized by the digitonin treatment. These data show that the membrane association of LANCL2 is influenced by the membrane cholesterol level and that actin displays a similar behavior in this respect.

To investigate the effect of cholesterol depletion on the subcellular distribution of LANCL2, we treated cells stably expressing LANCL2-GFP with mβCD. We noticed that the plasma membrane association of LANCL2-GFP remained unchanged (data not shown) but the cells easily detached from the matrix. A cell detachment assay showed that about 40% of the LANCL2-GFP expressing cells were released into the supernatant. Thus, the extent of detachment of the non-myristoylated mutant LANCL2-GFP mutG1A was about 50% of the membrane-associated actin and that actin displays a similar behavior in this respect.

3.5. Association of LANCL2 with the cortical actin cytoskeleton

The enhanced detachment of LANCL2 expressing cells suggested that LANCL2 is involved in the reorganization of the actin cytoskeleton and/or assembly of focal adhesions. Therefore, we performed fluorescence microscopy studies of LANCL2-GFP and actin that was visualized by rhodamine–phalloidin. LANCL2-GFP showed a striking co-localization with actin at the plasma membrane particularly the membrane protrusions (Fig. 6). To test the relevance of this co-localization, we incubated LANCL2-GFP expressing cells with the actin depolymerizing drugs, cytochalasin B and D. Again, we observed a strong co-localization, however, not equally distributed at the plasma membrane but in large aggregates that were predominantly found at blebbing sites (Fig. 6). It is to mention that cell membrane blebbing is a known phenomenon observed upon cytochalasin treatment. The co-localization data suggest a direct or indirect association of LANCL2 with the cortical actin cytoskeleton.

3.6. Overexpression of myristoylated LANCL2 increases cellular adriamycin sensitivity

Overexpressed LANCL2 has been found to sensitize MESSA cells to the anticancer drug adriamycin [8]. Therefore, we examined whether UAC cells overexpressing LANCL2 were similarly sensitive to adriamycin treatment. Cells were incubated...
with various concentrations of Adriamycin for 3 days and the cell viability was assessed by a proliferation assay. In GFP expressing cells, a rise of proliferation was observed at low concentrations of Adriamycin and a decrease at concentrations higher than 700 nM (Fig. 7). In contrast, LANCL2-GFP expressing cells showed a continuous decrease in cell viability in proportion to Adriamycin concentrations starting at 125 nM. At a concentration of 1 μM Adriamycin, the GFP expressing cells were still resistant and proliferated at a rate of 100% (compared to untreated GFP expressing cells), whereas the viability of LANCL2-GFP expressing cells was clearly diminished to 77% relative to untreated LANCL2-GFP cells (Fig. 7). To test whether the membrane association of LANCL2 is essential for this enhanced Adriamycin susceptibility, we treated the non-myristoylated mutant LANCL2-GFP mutG1A with the same Adriamycin concentrations. Interestingly, LANCL2-GFP mutG1A expressing cells did not show a decreased viability (Fig. 7) indicating that the membrane association of LANCL2 is important for the increased cellular Adriamycin sensitivity.

4. Discussion

In this study we show that the closely related proteins LANCL1 and LANCL2 (Fig. 1) display a different subcellular localization. Whereas LANCL1 is mainly a cytosolic and nuclear protein, a major part of LANCL2 is associated with the plasma membrane and juxta-nuclear vesicles that co-localize with the late endosomal markers Lamp1 and Lysotracker (data not shown). A minor part of LANCL2 is also present in the nucleus (Fig. 2). The membrane association is mediated by the LANCL2-specific N-terminal region which contains a
myristoylation site and a polybasic stretch. Because the membrane localization of LANCL2 is completely abolished by the treatment of the cells with the N-myristoyltransferase inhibitor HMA or when myristoylation is prevented by mutation (LANCL2-GFP mutG1A), this modification is essential for plasma membrane targeting of LANCL2. The non-myristoylated LANCL2 is found exclusively in the nucleus, particularly in nucleoli (Fig. 3).

It is known that myristoylated proteins have diverse biological functions in signal transduction under normal and pathological conditions [17,18]. N-myristoylation, which is a rare protein modification (http://mendel.imp.univie.ac.at/myristate/myrbase/), usually occurs co-translationally but post-translational myristoylation has also been reported [19,20]. David-Pfeuty et al. showed that myristoylation of pp60src regulates the functional localization and activity of this protein [21]. The authors suggest that myristoylation might prevent unregulated nuclear transport of proteins whose non-myristoylated counterparts are readily moved into the nucleus. This form of regulation might also apply to LANCL2.

Our biochemical data revealed that the N-terminal region of LANCL2 contains a PIP-binding motif with the polybasic region KRLKLH most probably representing the interacting site. LANCL2 preferentially interacts with PI monophosphates (Fig. 4). Apart from their function as signaling molecules, PIPs are known as mediators of the actin cytoskeleton membrane association [22,23]. Specifically, the redistribution of plasma membrane PI(4,5)P2 results in the reorganization of the cytoskeleton [22] and PI(5)P has been shown to be involved in cortical actin polymerization [23]. Cholesterol depletion affects the level of PI(4,5)P2 in the plasma membrane and thereby the membrane-associated cytoskeletal organization [22]. LANCL2 was released from isolated plasma membranes upon incubation with various cholesterol-depleting or -binding drugs together with components of the cortical cytoskeleton, actin and annexin II (Fig. 5A). It is conceivable that the cholesterol depletion interferes with the interaction between LANCL2 and PIPs thereby impairing the second membrane anchor of LANCL2. These data indicate that myristoylation and PIP-binding is crucial for high membrane affinity of LANCL2.

The co-localization of LANCL2 and actin at the plasma membrane and in large patches upon cytochalasin treatment suggests a close connection of LANCL2 to the cortical cytoskeleton (Fig. 6). Interestingly, cholesterol depletion of cells expressing the wild type LANCL2 but not the non-myristoylated mutant revealed enhanced cell detachment (Fig. 5B). Similarly, a myristoylation-dependent regulation of cell adhesion has been reported for the MARCKS proteins [24]. Membrane association of LANCL2 might have a regulatory function on the cortical cytoskeleton and thereby influence the cholesterol-dependent cell adhesion. However, more detailed analyses are needed to elucidate the mutual effects of myristoylation, PIP-binding, and membrane cholesterol levels on the membrane association of LANCL2 and its possible role in cortical cytoskeleton organization.

Park et al. reported that LANCL2 increases the sensitivity of MES-SA cells to the anticancer drug adriamycin by transcriptional suppression of the multi drug resistance protein P-gp [8]. Our respective data using LANCL2 overexpressing UAC cells (Fig. 7) confirm these results, however, the underlying mechanism appears to be different in this cell system. First, the UAC cells do not contain detectable amounts of P-gp (data not shown), and second, we observed that the enhancement of cellular sensitivity to adriamycin is critically dependent on the myristoylation of LANCL2 (Fig. 7). LANCL2 may be involved in a signaling pathway where its membrane association is an essential prerequisite. Several other myristoylated proteins have been shown to act as signaling components at the membrane [14]. It is also conceivable that a corresponding signaling pathway leads to the translocation of LANCL2 to the nucleus where it may exert the postulated transcriptional activity [8]. Alternatively, the increased sensitivity of LANCL2 overexpressing cells may be due to a regulatory effect on the cortical cytoskeleton dynamics or multi drug transport processes at the plasma membrane.

In this study, we have shown that LANCL2 is a novel myristoylated PIP-binding membrane protein that is found in association with the cortical actin cytoskeleton interfering with cell attachment in a cholesterol-dependent manner. Moreover, we have shown that LANCL2 increases the cellular adriamycin sensitivity only in the myristoylated and membrane-bound form. Further studies will have to clarify in detail how this protein is involved in these diverse processes.

Acknowledgments

This work was supported by grant number P15786 from the Austrian Science Fund (FWF).

We are grateful to Hemma Bauer for the LANCL1-GFP construct, purified LANCL1, advice, and discussions.

References