Identification of the amino acid sequence that targets peroxiredoxin 6 to lysosome-like structures of lung epithelial cells

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Am J Physiol Lung Cell Mol Physiol 297: L871–L880, 2009. First published August 21, 2009; doi:10.1152/ajplung.00052.2009.—Peroxiredoxin 6 (Prdx6), an enzyme with glutathione peroxidase and PLA2 (aiPLA2) activities, is highly expressed in respiratory epithelium, where it participates in phospholipid turnover and antioxidant defense. Prdx6 has been localized by immunocytochemistry and subcellular fractionation to acidic organelles (lung lamellar bodies and lysosomes) and cytosol. On the basis of their pH optima, we have postulated that protein subcellular localization determines the balance between the two activities of Prdx6. Using green fluorescent protein-labeled protein expression in alveolar epithelial cell lines, we showed Prdx6 localization to organel- lar structures resembling lamellar bodies in mouse lung epithelial alveolar epithelial cell lines, we showed Prdx6 localization to organel- lar sorting mechanism nor possible signals that target this protein to lamellar body and lysosomal compartments have been defined.

Our studies implicate the sequence between amino acids 31 and 40 in the Prdx6 NH2-terminal region as being essential for protein localization to organellar structures such as lamellar bodies and lysosomes. We determined that the role of this sequence in targeting is separate from its role in phospholipid binding. Thus this sequence appears to represent a unique signaling tag for determination of protein subcellular targeting.

METHODS

DNA constructs. The mammalian expression plasmid encoding NH2-terminal (GFP:Prdx6) green fluorescent protein (GFP)-tagged full-length Prdx6 has been described previously (24). The COOH-terminal (Prdx6-GFP)-tagged full-length protein was constructed by similar methods in the pGFP-N1 vector.

Deletion mutagenesis. A set of Prdx6:GFP COOH-terminal deletion mutants and one NH2-terminal deletion mutant were constructed using Pfu Turbo DNA polymerase, as described previously (33). Briefly, we used a pair of HPLC-purified 5′-phosphorylated inverse primers, separated by the region to be deleted, similar in size and melting temperature. The primer pairs used for mutagenesis are presented in Table 1. A mixture containing 150 ng of each primer, 50 ng of GFP:Prdx6 as a template DNA, 200 μM dNTPs, and 2.5 U of Pfu Turbo polymerase (Stratagene, La Jolla, CA) in a total volume of 50 μl was subjected to the PCR, characterized by the following conditions: denaturation at 95°C for 3 min, followed by 18 cycles of denaturation at 95°C for 45 s, annealing at 60–70°C, and extension at 68°C for 1 min/kb template. A portion of the reaction mixture (10 μl)
Lung alveolar epithelial cell line, were cultured in HITES medium (5). Cells were transfected with 3 μl of XL-Blue supercompetent cells (Stratagene). The Δ1–30 aa GFP-Prdx6 deletion mutant was used as template DNA to generate the GFP-tagged 31–40 aa construct.

Site-directed mutagenesis. Site-directed mutations were performed using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol using pairs of HPLC-purified primers (Table 1). In separate mutants of the full-length protein, S32 or H26 was substituted by alanine, or G30 and G34 were mutated to leucines. In the truncated 31–40 aa peptide, S32 was mutated to alanine, or H26 was substituted by alanine, or G30 and G34 were mutated to leucines.

Cell culture and transfections. Lung epithelial cell lines MLE-12 (CRL-2110) and A549 (CCL-185) were obtained from the American Type Culture Collection (Manassas, VA). MLE-12 cells (11), a mouse lung alveolar epithelial cell line, were cultured in HITES medium (5). Cells were transfected with 3 μl of Lipofectamine 2000 reagent (Invitrogen) per well according to the manufacturer’s protocol. Cells were subjected to experimental treatment of Lipofectamine 2000 reagent (Invitrogen) per well according to the manufacturer’s protocol. Cells were subjected to experimental treatment of Lipofectamine 2000 reagent (Invitrogen) per well according to the manufacturer’s protocol. Cells were subjected to experimental treatment of Lipofectamine 2000 reagent (Invitrogen) per well according to the manufacturer’s protocol.

A549 cells, a human lung carcinoma cell line (20), were grown in DMEM (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics. Although these cells possibly originated from an alveolar epithelial cell tumor, their lysosomes have relatively few of the characteristics of lamellar bodies. Cells were maintained in 5% CO2 at 37°C. For transient expression of GFP-tagged constructs in A549 cells, 95% confluent cell layers in six-well plates were transfected with 3 μg of each expression plasmid in 10 μl of Lipofectamine 2000 reagent (Invitrogen) per well according to the manufacturer’s protocol. Cells were subjected to experimental treatment 48 h after transfection.

Immunofluorescence and confocal microscopy. Cells cultured on glass coverslips were rinsed with PBS and either permeabilized by fixation with cold ethanol-acetone [1:1 (vol/vol)] for 5 min on ice or fixed with 3% paraformaldehyde for 10 min at room temperature and then permeabilized with 1% Triton X-100 for 10 min. Both methods gave similar results. The subcellular distribution of GFP:Prdx6 and its mutants in MLE-12 and/or A549 cells was observed under a confocal microscope (Bio-Rad) at ×60 magnification.

Nile Red and LysoTracker Red staining. Nile Red, a stain for lipid-rich organelles, was used to stain LBL structures in MLE-12 cells fixed in 3% paraformaldehyde (5). A saturated solution (0.1 mg/ml) of Nile Red (Sigma, St. Louis, MO) was prepared in acetone and stored with protection from light at −20°C. A working solution of Nile Red was prepared by addition of 1 ml of a 75:25 glycerol-water mixture to Nile Red stock solution (0.5 μl). Fixed GFP:Prdx6-transfected cells were subjected to 5 min of incubation at room temperature with 25 μl of the Nile Red working solution. LysoTracker Red, a stain for acidic compartments, was used as a lysosomal marker in A549 cells. LysoTracker Red (Invitrogen) was added to the GFP:Prdx6-transfected cells 30 min before fixation according to the manufacturer’s protocol. After they were stained, the cells were fixed,

Table 1. Pairs of oligonucleotides used to generate GFP-tagged Prdx6 deletion and point mutants by PCR

<table>
<thead>
<tr>
<th>Mutant</th>
<th>PCR Primers</th>
<th>Template Plasmid</th>
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<tr>
<td>1–70 aa</td>
<td>[Phos]-5′-CGGACTTGAGATTATGCTGAGATGCGG-3′</td>
<td>FL</td>
</tr>
<tr>
<td>1–40 aa</td>
<td>[Phos]-5′-TGAGGTGAGAGCTGCTGAGATGCGG-3′</td>
<td>FL</td>
</tr>
<tr>
<td>1–30 aa</td>
<td>[Phos]-5′-AAAGGTCGTTTGAATGCTGAGATGCGG-3′</td>
<td>FL</td>
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<tr>
<td>31–224 aa</td>
<td>[Phos]-5′-GATTTGATGCGGATTGTCTCTTGTCCACCGCAAGGAG-3′</td>
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<td>31–40 aa</td>
</tr>
<tr>
<td>31–34 aa</td>
<td>[Phos]-5′-CGGACTTGAGATTATGCTGAGATGCGG-3′</td>
<td>FL</td>
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GFP, green fluorescent protein; Prdx6, peroxiredoxin 6; FL, full-length Prdx6.

was then examined by agarose gel electrophoresis to determine whether the correct-sized product had been obtained. The rest of the reaction mixture was treated with 20 U of Dpn I restriction enzyme for 2 h at 37°C to digest parental DNA. The PCR product was purified from a 1% agarose gel using the QIAEX II gel extraction kit (Qiagen, Valencia, CA), ligated, and transformed into 50 μl of XL-Blue supercompetent cells (Stratagene). The Δ1–30 aa GFP-Prdx6 deletion mutant was used as template DNA to generate the GFP-tagged 31–40 aa construct.

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mounted on a slide, and observed with a confocal microscope using a ×60 objective.

Isolation of lysosome-like organelles. LBL structures from MLE-12 cells and lysosomes from A549 cells were isolated in isotonic sucrose as described previously (13). Briefly, pelleted cells (0.5 g) were washed once with PBS, resuspended in 5 ml of buffer containing 0.25 M sucrose in 10 mM Tris·HCl (pH 7.4), and homogenized with 10–15 strokes in a Teflon Dounce homogenizer. The cell debris was centrifuged at 12,000 g for 10 min at 4°C. CaCl₂ added to the cytosolic supernatant to a final concentration of 8 mM, and the sample was recentrifuged at 25,000 g for 15 min at 4°C. The pellet containing lysosomes or LBL structures was washed in 4 ml of 150 mM KCl in 10 mM Tris·HCl buffer (pH 7.4), and the organelles were resedimented by a final centrifugation at 25,000 g for 15 min at 4°C. Lamellar bodies also were isolated from mouse lung by sucrose gradient centrifugation as described previously (6). Prdx6 localization was analyzed by Western blot and flow cytometry analysis.

Flow cytometry analysis. Flow cytometry analysis of Prdx6 expression was performed in freshly isolated vesicles with a four-color dual-laser FACSCalibur system (Becton Dickinson, San Jose, CA) using CellQuest software as described previously (15, 19). Prdx6 was detected using a polyclonal primary antibody (17, 24) and FITC-conjugated goat anti-rabbit IgG secondary antibody (Jackson Laboratories, Bar Harbor, ME). Assays were developed in experimental pairs (surface and luminal Prdx6 expression) as single-color staining (26). For assay of luminal Prdx6, the freshly isolated organellar fraction was treated with 0.1% paraformaldehyde for 20 min, washed three times in PBS, and lightly permeabilized with 0.1% Triton X-100 at room temperature. Samples were incubated with antibodies for 1 h on ice and then washed three times in PBS before flow cytometry. Extraluminal (surface) Prdx6 was detected by staining without prior permeabilization. Both unstained vesicles and incubation without the primary antibody were evaluated as negative controls.

Western blot analysis. Isolated lamellar bodies or lysosomal vesicles were permeabilized with 0.1% Triton X-100 for 30 min on ice to extract luminal proteins. Vesicular membranes were pelleted at 100,000 g and then treated with 0.2 M Na₂CO₃ for an additional 30 min on ice to detach proteins loosely associated with lysosomal membranes. Luminal and membrane fractions were subjected to Western blot analysis using the two-color Odyssey LI-COR (Lincoln, NE) technique as previously described (25). The secondary antibody IRDye 800 goat anti-rabbit (Rockland, Gilbertsville, PA) was used for imaging in the green 800-nm channel.

Liposome preparation. Unilamellar liposomes consisting of DPPC-egg phosphatidylcholine-cholesterol-phosphatidylglycerol (50:25:15:10, mol/mol) were prepared by extrusion under pressure, as described previously (7). This lipid mixture was chosen to mimic the composition of lung surfactant. Fluorescently labeled liposomes were prepared by replacement of 2 mol% of egg phosphatidylcholine with N-(5-dimethylaminonaphthalene-1-sulfonyl)-sn-glycero-3-phosphatidylethanolamine (N-DNS-PE). Analysis by dynamic light scattering (DLS 90 Plus Particle Size Analyzer, Brookhaven Instruments, Hollis, NY) showed a homogeneous population of 100- to 120-nm-diameter liposomes, which represented >95% of total vesicles.

Fluorescence measurements. N-DNS-PE-labeled phospholipids were used to study the interaction between phospholipids and the Prdx6 31–40 aa region, which was measured as the change in fluorescence emission as described previously (22). All fluorescence studies were done at 22 ± 0.5°C with use of a water bath temperature-controlled sample holder. The ratio of fluorescence emission at 415 nm to fluorescence emission at 505 nm was measured with a spectrophuorometer equipped with a single-photon counting system for fluorescence intensity detection, dual fluorescence, and absorbance channels with use of excitation and emission slits of 1 and 2 nm, respectively (Photon Technology International, Lawrenceville, NJ).

Binding of Prdx6 peptides to liposomes. Synthetic decamer peptides, corresponding to the Prdx6 25–34 aa and 31–40 aa sequences, were produced and purified by ELIM Biopharmaceuticals (San Francisco, CA). Four peptides were synthesized: two corresponded to the wild-type sequences for amino acids 25–34 (FHDFLGDSWG) and amino acids 31–40 (DSWGILFSHP), and the others were the H26A and S32A mutants of these peptides. The COOH-terminal amino acid (proline) in the 31–40 aa sequence was substituted with lysine. Peptides were NH₂ terminally acetylated. Real-time determination of the binding of Prdx6 wild-type and mutant peptides (100 μM final concentration) to unilamellar liposomes (100 μM total phospholipids in 40 mM potassium phosphate buffer (pH 7.4) or in 40 mM sodium acetate buffer (pH 4.0)) was performed in a time-based ratiometric mode of the fluorometer, with recording of one measurement per second for 15 sec before and 45 min after the addition of the peptide as described previously (22). Excitation was set at 340 nm, and fluorescence emission was measured at 415 and 505 nm, with constant stirring of the sample before and after the addition of the peptide. The data were plotted as the ratio of fluorescence emission at 415 nm to fluorescence emission at 505 nm and analyzed using standard sigmoidal curve fitting (4 parameters) with SigmaPlot version 9 software (SPSS, Chicago, IL). R² for all fits was >0.98.

Statistical analysis. Values are means ± SE. Statistical significance was assessed with SigmaStat software (Jandel Scientific, San Jose, CA). Group differences were evaluated by one-way ANOVA or by Student’s t-test as appropriate. Differences between mean values were considered statistically significant at P < 0.05.

Fig. 1. Expression of endogenous peroxiredoxin 6 (Prdx6) in lysosome-related compartments in lung epithelial cells studied by flow cytometry and Western blotting. A: flow cytometry analysis of Prdx6 content in lysosome-related organelles before and after light permeabilization. Gray peak, surface staining; black peak, luminal expression; dashed peak, negative control; au, arbitrary units. B: Western blot analysis of Prdx6 expression in lysosomes freshly isolated from mouse lung epithelial (MLE-12) and A549 cells and in mouse lung lamellar bodies. Organelles were treated with Na₂CO₃ to differentiate between luminal and intrinsic membrane protein. Lysosome-associated membrane protein (LAMP-1) was used as a lysosomal membrane marker.
RESULTS

Prdx6 localization in lysosome-like organelles. Our previous studies demonstrated Prdx6 expression in lysosomes and lamellar bodies of lung alveolar type II cells (1, 10, 35). To further investigate Prdx6 subcellular localization, we isolated lamellar bodies from mouse lung homogenate, LBL organelles from MLE-12 cells derived from mice, and lysosomes from human lung (A549) epithelial cells (Fig. 1). We have termed the isolates from MLE-12 cells LBL structures and those from A549 cells lysosomes, although they have similarities and may represent different stages in development of the secretory bodies. Prdx6 staining, as assessed by flow cytometry, increased significantly after light permeabilization, indicating that it is within the organelles (Fig. 1A). Identity of the organelles was confirmed by staining with lysosome-associated membrane protein (LAMP-1; Fig. 1B), a marker of lysosome-related compartments (14). The organelles were treated with Na2CO3 and then subjected to Western blot analysis to differentiate proteins that were loosely associated with the membrane (luminal proteins) from intrinsic membrane proteins. These studies showed that Prdx6 is predominantly localized to the lumen of the lysosomal vesicles (Fig. 1B).

Prdx6:GFP constructs were prepared for study of the mechanism of Prdx6 targeting. As a first step, we expressed these constructs or GFP alone in MLE-12 and A549 cells, cell lines derived from mouse and human lung epithelium, respectively, and evaluated their targeting to lysosome-related compartments. In the MLE-12 and A549 cells, the expression of GFP was confirmed by staining with lysosome-associated membrane protein (LAMP-1; Fig. 1B), a marker of lysosome-related compartments (14). The organelles were treated with Na2CO3 and then subjected to Western blot analysis to differentiate proteins that were loosely associated with the membrane (luminal proteins) from intrinsic membrane proteins. These studies showed that Prdx6 is predominantly localized to the lumen of the lysosomal vesicles (Fig. 1B).

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Fig. 3. Expression of GFP-tagged Prdx6 deletion mutants in MLE-12 and A549 lung epithelial cells. A: schematic representation of GFP:Prdx6 deletion constructs. B: colocalization of each of the GFP:Prdx6 deletion mutants with Nile Red in MLE-12 cells. C: colocalization of Prdx6 with LysoTracker Red in A549 cells. Left panels: fusion constructs expressed in transfected cells. Middle panels: lamellar bodies (B) and lysosomes (C), shown in red. Right panels: colocalization, detected by yellow color, in the merged image. Inset in C demonstrates the absence of colocalization of GFP:Prdx6 (1–30 aa) in lysosomes.
alone was diffuse, compatible with cytoplasmic localization (Fig. 2, A and B, top panels). On the other hand, GFP:Prdx6 colocalized with Nile Red, a marker for lamellar bodies, in MLE-12 cells (Fig. 2A, middle and bottom panels) and with the lysosomal marker LysoTracker Red in A549 cells (Fig. 2B). Targeting was similar for the GFP:Prdx6 and the Prdx6:GFP constructs, indicating that location of the tag did not affect localization (Fig. 2C). These results indicate that an element in Prdx6 can direct the Prdx6:GFP fusion complex to acidic organelles.

**Prdx6 lysosomal targeting signal.** To identify a possible amino acid sequence responsible for targeting of Prdx6 to lamellar bodies and lysosomes, we generated a set of GFP-tagged Prdx6 deletion mutants (Fig. 3A). In MLE-12 (Fig. 3B) and A549 (Fig. 3C) cells, deletion of the COOH-terminal region had no effect, as indicated by normal targeting of the peptides composed of amino acids 1–70 and 1–40 of Prdx6. On the other hand, a peptide of amino acids 1–30 fused to GFP did not show targeting. This peptide appeared to be toxic for the cells, deletion of the COOH-terminal C tag showed targeting similar to the full-length protein (Fig. 3B). These same point mutations within the 1–40 aa peptide, as well as the S32A mutation in the GFP:31–40 aa fusion peptide, also abolished targeting to lysosomal organelles in MLE-12 cells (Fig. 4C). Of course, the G30 amino acid is not contained in the 31–40 aa targeting sequence. Thus these results indicate that S32 and G34 within the 31–40 aa targeting sequence are required for Prdx6 subcellular localization. The S38L (Fig. 4D) and S38G (not shown) mutations had no effect, and thus this moiety does not play a role in targeting. We chose these substitutions for the S38 mutation, because serine and glycine appear to be interchangeable in the putative targeting motif (thereby serving as a control) whereas the leucine mutation of G34 resulted in loss of targeting.

H26, like S32, in Prdx6 is a component of the PLA2 catalytic triad and also has been shown to participate in binding of Prdx6 to phospholipid substrate (22). To evaluate the possible role of phospholipid binding in Prdx6 targeting, we determined the effect of mutating H26 (H26A) in the full-length protein. This mutation had no effect on targeting, indicating that phospholipid binding is not required for Prdx6 organellar localization (Fig. 4D).

**Binding of synthetic peptides to liposomes.** To provide additional evidence related to the negative results for a role of phospholipid binding, we evaluated the binding of peptides to phospholipids in vitro. The N-DNS-PE probe was used to label the lipid shell of liposomes, and the binding of Prdx6 25–34 aa and 31–40 aa peptides to liposomes was studied by recording the real-time changes in the 415-nm to 505-nm fluorescence emission ratio (Fig. 5). These studies were done at pH 4.0 and 7.4 on the basis of our previous results with the full-length protein (22, 23). Shift of the fluorescence emission maximum with the N-DNS-PE probe from 505 to 415 nm in the presence of the peptide reflects the change in the polarity of its surroundings, indicating the shielding of the DNS chromophore by bound ligand (22). Thus an increase in the 415-nm to 505-nm fluorescence emission ratio indicates binding of the Prdx6 peptide to the liposomal surface. There was significant binding of the wild-type peptide to N-DNS-PE at pH 4.0 but significantly less binding at pH 7.4 (Fig. 5, A and B). The 25–34 aa peptide bound to a lesser degree at pH4 and did not bind at pH 7.4. The effect on binding of the H26A and S32A mutations in the Prdx6 25–34 and 31–40 aa sequences was studied. Compared with the respective wild-type peptides, both mutant peptides showed a significant reduction in binding (Fig. 5, Table 2). These data regarding the effect of pH and the
their localization in type II epithelial cells. Furthermore, the LBL structures (MLE-12) and lysosomes (A549) similar to express endogenous Prdx6 in the luminal compartment of their readily transfectable cells that express Prdx6. These cell lines MLE-12 and A549 cell lines derived from lung epithelium as compartments in lung type II epithelial cells. We studied the regulate the subcellular targeting of Prdx6 to lysosome-related mixture.

![Graph A](image1)

**Fig. 5.** Binding of Prdx6 25–34 aa and 31–40 aa peptides to N-(5-dimethylaminomethyl-4-sulfonyl)-3-glyceryl-3-phosphatidylethanolamine (N-DNS-PE) unilamellar liposomes. Liposomes (100 µM total phospholipid) were suspended in 40 mM sodium acetate buffer (pH 4.0) or 40 mM potassium phosphate buffer (pH 7.4). Binding was determined by measurement of the ratio of fluorescence emission at 415 nm to fluorescence emission at 505 nm. Binding curves of wild-type (WT) peptides and S32A and H26A mutants (100 µM) to N-DNS-PE liposomes at pH 4.0 and pH 7.4 are shown by a sigmoidal curve fit. Binding is shown in real time by detection of the DNS fluorescence ratio (415/505 nm). Arrow indicates addition of peptides to the liposome mixture.

![Graph B](image2)

**DISCUSSION**

The goal for this study was to evaluate mechanisms that regulate the subcellular targeting of Prdx6 to lysosome-related compartments in lung type II epithelial cells. We studied the MLE-12 and A549 cell lines derived from lung epithelium as readily transfectable cells that express Prdx6. These cell lines express endogenous Prdx6 in the luminal compartment of their LBL structures (MLE-12) and lysosomes (A549) similar to their localization in type II epithelial cells. Furthermore, the Prdx6:GFP fusion protein (with the GFP tag on the NH2 or COOH terminus of Prdx6) in these cell lines is targeted to organelles that co-stain with markers for lysosome-like structures. This targeting was not a default pathway, since it was abolished by site-specific mutations in the protein.

Our initial focus was on the primary sequence of Prdx6, inasmuch as it is known that discrete domains located within the amino acid sequence of proteins can determine subcellular localization. We determined that deletion of amino acids 31–40 prevented the lysosomal localization of Prdx6 and that this 10-aa peptide by itself could drive lysosomal targeting of GFP. Thus this peptide appears to be necessary and sufficient for Prdx6 subcellular localization. A smaller fragment (amino acids 31–34) was ineffective as a targeting signal. The 31–40 aa sequence is 100% identical in human, rat, mouse, and bovine Prdx6 (9, 17).

Surfactant protein C (SP-C) is synthesized by type II alveolar epithelial cells and, similar to Prdx6, is targeted to lysosome-like organelles/lamellar bodies. However, in contrast to the luminal Prdx6, SP-C is an integral membrane protein, and its topography has an important role in its targeting (27). Also in contrast to Prdx6, the “purpose” for targeting of SP-C is for further processing before secretion, whereas the role of Prdx6 targeting is expression of enzymatic activity within the organelles. The targeting sequence for SP-C has been identified as a 9-aa peptide, MESPPDYSA, present at the NH2 terminus of the protein (16). Neither this specific sequence nor homologous domains are present in Prdx6.

Previous studies in cells from other organs have shown that protein targeting to post-Golgi compartments such as endosomes, lysosomes, or lysosome-like organelles may involve tyrosine-based YXX0-type motifs, where 0 is a hydrophobic group amino acid, such as glycine, alanine, valine, leucine, or isoleucine (30, 32). [D/E]XXX[L/I] and DXXLL-type sequences also have been shown to mediate the rapid internalization and targeting of proteins to endosomes and lysosomes (30). These dileucine sorting motifs require the presence of two consecutive leucines or a leucine-isoleucine pair. Within the Prdx6 amino acid sequence, a YXX0-type motif, YNGA, is located between amino acids 89 and 92 and a dileucine motif can be found between amino acids 107 and 112. However, in this study, we demonstrate that the deletion of all residues beyond amino acid 40 and before amino acid 30 of Prdx6 has no effect on targeting. Therefore, these tyrosine-based and dileucine motifs are unlikely to be responsible for lysosomal localization of Prdx6.

Three recognized motifs within the Prdx6 30–40 aa sequence (GDSWGILFSHP) could be involved with targeting. The first is a consensus lipase motif (GXSXG) (18, 31), which is necessary for PLA2 activity of the protein and appears to be crucial for Prdx6 binding to phospholipid substrate (22, 23). In previous studies, Prdx6 bound to phospholipids in mixed unilamellar liposomes at pH 4.0 but bound poorly at pH 7.4, which is likely to be closer to the pH of the organelles (presumably, the Golgi) where targeting is initiated. The present studies using a synthetic peptide (amino acids 31–40) is a hydrophobic

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<th>Peptide</th>
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<tr>
<td>31–40 aa</td>
<td>100</td>
<td>33.7 ± 1.8</td>
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<tr>
<td>S32A</td>
<td>28.2 ± 1.5</td>
<td>14.1 ± 0.1</td>
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<tr>
<td>25–34 aa</td>
<td>42.8 ± 1.6</td>
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<td>H26A</td>
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Values are means ± SE. WT, wild type.
containing the lipase motif gave results for binding that are similar to previous observations with the full-length protein. Binding was greatly diminished by mutation of S32 within the lipase motif (22, 23). Mutation of S32 also abolished binding, raising the possibility that binding of Prdx6 to lipids may be the mechanism for organellar localization. Lipid binding by the protein also is diminished by mutation of H26, another component of the catalytic triad that is necessary for PLA2 activity (22). The present results with a synthetic peptide (Fig. 5) confirmed a role for H26 in binding. However, this mutation had no effect on targeting of the full-length protein (Fig. 4). This result indicates that lipid binding is not necessary for Prdx6 targeting.

The 30–40 aa sequence of Prdx6 also contains two signature GXXGX/S-type motifs, known as glycine zippers [GDSWG (amino acids 30–34) and GILFS (amino acids 34–38)]. A GXXGX-type signal has been demonstrated to serve as the framework for dimerization of transmembrane α-helices (29), thus serving to target proteins to the plasma membrane (12). A similar mechanism was proposed to mediate the function of membrane receptors, where GXXGX-like motifs were found to be present in transmembrane domains of many G protein-coupled receptors. These include the α-factor receptor of various yeast species, class A amine and cannabinoid receptors, class B secretin-like receptors, and class C metabotropic receptors (28). Mutation within the GXXGX motif was shown to reduce expression and increase receptor retention in intracellular compartments such as the endoplasmic reticulum. The mechanisms for this effect are unknown. Mutation of G34, along with G30, did abolish targeting of the GFP:Prdx6 construct; however, the effective targeting sequence was amino acids 31–40. Thus the first glycine (G30) is not essential for Prdx6 targeting. With respect to the second glycine zipper motif, mutation of S38 had no effect on targeting. These results indicate that the glycine zipper signaling sequences per se do not have a role in Prdx6 subcellular targeting.

In summary, we have evaluated mechanisms of Prdx6 targeting to specific acidic subcellular locations (lysosomes and lamellar bodies) that are dependent on the PLA2 activity of the protein. Our data indicate that protein localization in lysosome-related compartments depends on the 31–40 aa NH2-terminal region of Prdx6. Within this sequence, the amino acids S32 and G34 and at least one or more of amino acids 35–40 play an essential role. On the basis of these results, the minimal Prdx6 lysosomal targeting motif could be as small as SxGx, whereas the maximal motif is SxSxG(x)2x(x)2, where x represents amino acids that have not yet been studied for their essentiality and X is any amino acid. This study has not evaluated mechanisms for the cytosolic localization of Prdx6, which presumably is necessary for its glutathione peroxidase activity. We expect that Prdx6 synthesis begins on free ribosomes in the cytosol. We propose that much of the newly synthesized Prdx6 remains cytosolic, whereas some fraction is internalized into the lysosomal matrix, possibly through interaction with a chaperone protein. Although the present results indicate the signal sequence necessary for lysosomal localization, further studies are necessary for an understanding of the mechanism for regulation of Prdx6 distribution between cytoplasmic and lysosomal compartments.

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