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Characterization of a Di-leucine–based Signal in the Cytoplasmic Tail of the Nucleotide-pyrophosphatase NPP1 That Mediates Basolateral Targeting but not Endocytosis

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Enzymes of the nucleotide pyrophosphatase/phosphodiesterase (NPPase) family are expressed at opposite surfaces in polarized epithelial cells. We investigated the targeting signal of NPP1, which is exclusively expressed at the basolateral surface. Full-length NPP1 and different constructs and mutants were transfected into the polarized MDCK cell line. Expression of the proteins was analyzed by confocal microscopy and surface biotinylation. The basolateral signal of NPP1 was identified as a di-leucine motif located in the cytoplasmic tail. Mutation of either or both leucines largely redirected NPP1 to the apical surface. Furthermore, addition of the conserved sequence AAASLLAP redirected the apical nucleotide pyrophosphatase/phosphodiesterase NPP3 to the basolateral surface. Full-length NPP1 was not significantly internalized. However, when the cytoplasmic tail was deleted upstream the di-leucine motif or when the six upstream flanking amino acids were deleted, the protein was mainly found intracellularly. Endocytosis experiments indicated that these mutants were endocytosed from the basolateral surface. These results identify the basolateral signal of NPP1 as a short sequence including a di-leucine motif that is dominant over apical determinants and point to the importance of surrounding amino acids in determining whether the signal will function as a basolateral signal only or as an endocytotic signal as well.

INTRODUCTION

The regulation of the concentration of nucleotides and nucleosides in the extracellular medium depends on the cooperation of a variety of ectonucleotidases. The ectonucleotide pyrophosphatase/phosphodiesterase (E-NPP) family comprises cell surface enzymes capable of hydrolyzing phosphodiester bonds of nucleotides and nucleic acids and pyrophosphate bonds of nucleotides and nucleotide sugars (Bollen *et al.*, 2000; Goding, 2000). Three members have been identified, which are now called NPP1, NPP2 and NPP3, in the order they have been cloned (Zimmerman *et al.*, 2000). Recently, 2 additional members (putative NPP4 and NPP5) have been identified (Gijsbers *et al.*, 2001). There is evidence that the NPP family is related to alkaline phosphatase in structure and function (Galperin *et al.*, 1998; Bollen *et al.*, 2000; Gijsbers *et al.*, 2001).

The first member of the family, NPP1, was initially called PC-1. PC-1 was first identified as a marker of terminally differentiated B cells (plasma cells) within the lymphoid system. However, it was later shown to be expressed by many cell types including chondrocytes, osteoblasts, hepatocytes, and epithelial cells of the epididymis (Harahap and Goding, 1988). The precise function of the enzyme in different cells is not fully known. A fundamental role in bone formation has recently been shown by the phenotype of ttw/ttw (tiptoe walking) mice, which have a nonsense mutation in PC-1 sequence resulting in the loss of more than one-third of the molecule and the generation of knock out mice (Okawa *et al.*, 1998; Sali *et al.*, 2000). These mice have excessive bone formation around growth plates, cartilage, and tendons. It has also been proposed that overexpression of PC-1 is causally associated with extreme insulin resistance

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Abbreviations used: AP-1: adaptor protein 1; AP-2: adaptor protein 2; GFP: green fluorescent protein; NPP1: nucleotide pyrophosphatase/phosphodiesterase 1; NPP3: nucleotide pyrophosphatase/phosphodiesterase 3; PC-1: plasma cell antigen 1.

in diabetes mellitus (Maddux *et al.*, 1995), although these findings remain controversial (Whitehead *et al.*, 1997).

NPP2 has been named autotaxin, a tumor cell motility factor secreted by a human melanoma cell line. cDNA cloning of autotaxin revealed that the protein was a cleaved form of an integral membrane protein that shared 45% identity with PC-1 (Murata *et al.*, 1994). The third member, NPP3, which has been cloned under the names gp130^{rb13-6}, B10, and PD1β, also shares ~50% identity with PC-1 (Deissler *et al.*, 1995; Jin-Hua *et al.*, 1997; Scott *et al.*, 1997). NPP1–3 are type II transmembrane glycoproteins with short cytoplasmic tails and large extracellular domains bearing the conserved enzymatic site, whereas NPP4 and NPP5 appear to be type I membrane proteins (Gijsbers *et al.*, 2001).

The existence of several enzymes with similar activity and broad specificity suggests that their biological functions may vary according to the tissue in which they are expressed and to their cellular location. An interesting finding was that certain cells express several of these enzymes, but in different places. This was shown in the case of rat hepatocytes, which express NPP1 at the basolateral (sinusoidal) surface and NPP3 exclusively at the apical (canalicular) surface (Scott *et al.*, 1997). Such restricted locations suggest that these enzymes perform specialized functions at each pole in epithelial cells and imply that each molecule has acquired specific signals for their targeting to the apical or basolateral surface. Remarkably, the cytoplasmic domains show only patchy and discontinuous conservation between human and mouse, and it seems likely that the conserved "islands" in the cytoplasmic tails contain targeting signals (Goding, 2000). Therefore, NPP1 and NPP3 are interesting model proteins in order to study the molecular bases of polarized targeting in epithelial cells.

In the present study, we searched for the basolateral targeting signal of NPP1 by analyzing the polarized distribution of the wild-type protein and of different constructs or engineered mutants transfected in the polarized MDCK cell line. We identify the signal as a short cytoplasmic amino acid sequence including a conserved di-leucine motif, which appears to mediate basolateral targeting only and not endocytosis. However, we show that the di-leucine motif is able to behave as an endocytosis signal when upstream amino acids are deleted. This signal is autonomous and dominant over apical determinants because it is able to redirect the apical protein NPP3 to the basolateral surface.

MATERIALS AND METHODS

Constructs

The mouse NPP1 wild type was cloned between the *Sal*I and *Not*I sites of the polylinker of the pCI-neo vector (Promega, Charbonnieres, France). The rat NPP3 wild type was cloned into the pCI-neo vector as described previously (Rajho Meerson *et al.*, 2000; Figure 1A).

The constructs were engineered with the use of a pCI-neo vector mutated to remove a *Bam*HI site located just downstream the 3′ region of the synthetic poly A of the neomycin resistance gene. The basic approach was to create a series of cassettes encoding the cytoplasmic/transmembrane domain and the extracellular domains, such that they could be ligated together in various combinations. The cytoplasmic/transmembrane domain and the ectodomain of the mouse NPP1 cDNA were separately amplified by PCR with the use of pairs of primers designed to create a *Bam*HI site between the transmembrane and the ectodomain, and inserted between *Nhe*I and *Eco*RI sites of the pCI-neo polylinker. The same strategy was used to create a *Bam*HI site just after the transmembrane domain of NPP3. In each case, creation of the *Bam*HI site changed the amino acid sequence at the junction of the transmembrane and extracellular domains from Lys-Pro to Glu-Pro, but this had no effect on targeting. Except when indicated, the inserts described below were cloned into the pCI-neo (*Bam*HI−) vector containing NPP1 previously digested with *Nhe*I and *Bam*HI to delete the cytoplasmic and the transmembrane domains and with the use of the same sites. All the PCR reactions amplifying the NPP1 or NPP3 cytoplasmic/transmembrane domains were performed with the use of mouse NPP1 or rat NPP3 in pBluescript II SK+ as template.

The cytoplasmic/transmembrane domain of NPP1 was amplified by PCR and either ligated with E-GFP previously amplified separately by PCR to generate an in-frame *Bam*HI site compatible with the *Bam*HI site created in the transmembrane/cytoplasmic cassette as described above, and cloned into the *Nhe*I-*Eco*RI sites of pCI-neo vector wild type to obtain CytoTmNPP1/EctoGFP or directly inserted upstream the ectodomain of NPP3 to obtain CytoTmNPP1/EctoNPP3 (Figure 1B).

The strategy of PCR-ligation-PCR (Ali and Steinkasserer, 1995) was used to construct the chimeras CytoNPP1/TmEctoNPP3 and CytoNPP3/TmEctoNPP1 (Figure 1B). The first step consisted of separate amplification of the cytoplasmic tails and of the transmembrane domains of mouse NPP1 or rat NPP3. The PCR products were then phosphorylated. The NPP1 cytoplasmic tail was blunt ligated with the transmembrane domain of NPP3 and the NPP3 cytoplasmic tail was blunt ligated with the transmembrane domain of NPP1. A second series of PCR allowed amplification of the ligated cytoNPP1-TmNPP3 and cytoNPP3-TmNPP1 with the use of the 5′ upstream and 3′ downstream primers of the first series of PCR. Finally, the products were purified and cloned, respectively, upstream of the NPP3 ectodomain (CytoNPP1/TmEctoNPP3) between *Xho*I and *Bam*HI sites and upstream of the NPP1 ectodomain (CytoNPP3/TmEctoNPP1) between *Nhe*I and *Bam*HI sites.

Deletion mutants NPP1/Δ2-5, NPP1/Δ1-34, NPP1/MLL, NPP1/MLA, and NPP1/MAL (Figure 1, C and D) were obtained by PCR amplification with the use of the same reverse primer complementary to the 3′ end of NPP1 transmembrane domain. The divergent forward primers were designed 1) to induce a deletion of the first 12 nucleotides corresponding to the ERDG motif located in the N-terminal part of the NPP1 cytoplasmic domain (NPP1/Δ2-5) 2) to initiate the translation at the second methionine Met^{35} (NPP1/Δ1-34), at the leucines Leu^{31}-Leu^{32} (NPP1/MLL) ,or at the mutated motif Leu-Ala (NPP1/MLA) or Ala-Leu (NPP1/MAL).

Point mutations of Leu^{31} and Leu^{32} of the full-length NPP1 (NPP1/LA and NPP1/AL) were obtained with the use of the Gene Editor In Vitro Site Directed Mutagenesis kit (Promega) according to manufacturer's instructions with NPP1 in pBluescript SK+ as template (Figure 1E). Then a PCR was performed to add restriction sites at each extremity of the mutated cytoplasmic tail and the transmembrane domain of NPP1, and the product consisting of the mutated cytoplasmic tail and wild-type transmembrane region was inserted upstream the ectodomain of NPP1.

The double mutant NPP1/AA construct (Figure 1E) was obtained by a PCR-ligation-PCR method (Ali and Steinkasserer, 1995). A first PCR was performed with the use of a pair of primers allowing the separate amplification of the N-terminal (5′) part of the cytoplasmic tail with the use of a reverse primer containing a mutation creating two alanines instead of the original two leucines at positions 31 and 32. A second pair of primers was used to amplify the 3′ half of the cytoplasmic tail and the transmembrane domain of NPP1. The PCR products were phosphorylated and ligated together, and a final PCR was performed to produce the mutated cytoplasmic tail and the transmembrane domain, with appropriate restriction sites for insertion into the pCI-neo vector, upstream the ectodomain of NPP1,

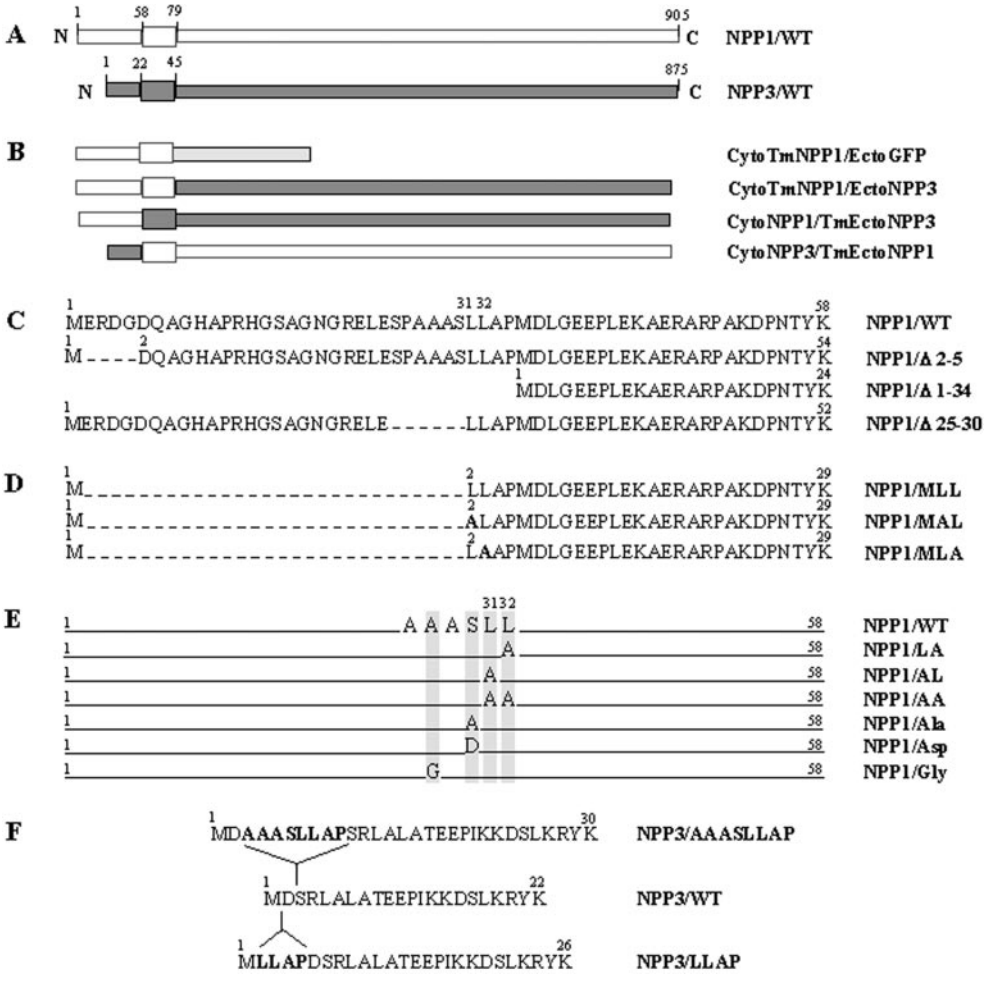

Figure 1. Schematic representation of wild-type proteins and constructs. (A) NPP1 and NPP3 wild-types. Numbers indicate the limits between the cytoplasmic, transmembrane and ectodomains. (B) Chimeras. In the CytoTmNPP1/EctoGFP and CytoTmNPP1/EctoNPP3 chimeras the ectodomain of NPP1 is replaced either by GFP or by the ectodomain of NPP3. The CytoNPP1/TmEctoNPP3 chimera is made of the cytoplasmic domain of NPP1 and the transmembrane and ectodomain of NPP3. The CytoNPP3/TmEctoNPP1 chimera combines the cytoplasmic domain of NPP3 and the transmembrane and ectodomain of NPP1. (C) Amino acid sequence of the cytoplasmic domain of NPP1/WT, NPP1/Δ2-5 deleted of residues 2–5, NPP1/Δ1-34 deleted of the first 34 residues and NPP1/Δ25-30 deleted of residues 25–30. (D) Amino acid sequence of the cytoplasmic domain of NPP1/MLL that is deleted from amino acids 2–30 and the mutants NPP1/MAL and NPP1/MLA. Alanine substitutions are in boldface. (E) Point and double mutations of leu[31]-leu[32] (NPP1/LA, NPP1/AL, NPP1/AA) and point mutations of Ser[30] (NPP1/Ala, NPP1/Asp), and Ala[28] (NPP1/Gly) in full-length NPP1. (F) Amino acid sequence of the cytoplasmic domain of NPP3/WT and of the NPP3/AAASLLAP and NPP3/LLAP chimeras which respectively contain the additional amino acid stretches AAASLLAP and LLAP indicated in bold.

with the use of the *Bam*HI site at the junction of the transmembrane and extracellular domains as previously described.

Deletion mutant NPP1/Δ25-30 (Figure 1C), point mutations of Ser[30] (NPP1/Ala, NPP1/Asp), or Ala[28] (NPP1/Gly; Figure 1E) as well as insertion of motifs AAASLLAP or LLAP at the N-terminal extremity of the cytoplasmic tail of NPP3 (NPP3/AAASLLAP and NPP3/LLAP; Figure 1F) were obtained with the use of the Quick Change Site Directed Mutagenesis Kit (Stratagene Europe, Amsterdam Zvidoost, The Netherlands). All the constructs were verified by sequencing.

Cell Culture and Transfection

MDCK cells (strain II) were grown at 37°C in DMEM (Life Technologies Inc, Cergy-Pontoise, France) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin, with a 10% CO_2/air atmosphere. The medium was changed every day. Cells were transfected with the use of a cationic lipid (Fugene 6; Roche, Meylan, France) according to the supplier's protocol. Cells were seeded in 6-well plates (1×10^5 cells per well), and transfection was performed the following day. Six microliters of Fugene 6 and 2 μg of plasmid DNA were mixed and left at room temperature for 15 min to allow complex formation. The DNA-lipid complex was then added to the cells. After 48 h of transfection, cells were selected with 1 mg/ml G418 (Life Technologies). Cells were screened by indirect immunofluorescence or by direct fluorescence of GFP as described below.

In several cases, the transfected cell population was enriched in positive cells by magnetic labeling (Miltenyi Biotec, Paris, France). Briefly, cells were trypsinized, incubated first with a mAb IR 518 anti-mouse NPP1 (ascites diluted 1:400 in PBS-BSA) for 30 min at 4°C, and then incubated with rat anti-mouse IgG1 magnetic microbeads for 15 min at 4°C. Finally, the cells were applied to a separation column placed in a magnetic field. Magnetically labeled positive cells retained in the column were eluted with culture medium and expanded.

For microscopy and biochemistry experiments, cells were grown to confluence for 10 d on Transwell polycarbonate filters units (0.4-μm pore size) of 12 and 24 mm diameter, respectively (Costar Corp., Cambridge, MA). The formation of tight monolayers was monitored by the difference between the level of the apical and basolateral media and confirmed by measuring transepithelial resistance with Millicell-ERS electrodes (Millipore Corporation, Bedford, MA). Before each experiment, cells were treated for 24 h with 10 mM sodium butyrate (Sigma, St. Quentin Fallavier, France), which induces the cytomegalovirus promoter transcriptional activity; this treatment increased expression but did not change the expression polarity of the transgenes.

Indirect Immunofluorescence and Confocal Microscopy

Filter-grown cells were fixed with 2% paraformaldehyde in PBS, pH 7.4, for 20 min at room temperature. Cells were then incubated for 15 min with 50 mM ammonium chloride and 0.1% BSA in PBS and permeabilized with 0.075% saponin for 15 min at room temperature. After three washes in PBS/BSA/NH_4Cl, cells were incubated for 1 h at room temperature with mAb B10 anti-rat NPP3 (ascites diluted 1:250 in PBS-BSA) or with mAb IR 518 anti-mouse NPP1 (ascites diluted 1:400 in PBS-BSA) in a humidified chamber. Cells were washed with PBS-BSA and incubated with FITC-conjugated species-specific donkey anti-mouse IgG antibody diluted 1:200 (Interchim, Lyon, France). After three washes with PBS-BSA, cells were incubated with 1 mg/ml RNAse A (Sigma) for 10 min and then with 2.5 μg/ml propidium iodide for 1 min to stain the nuclei. Cells were rinsed three times with PBS-BSA and incubated with 1,4-diazabicyclo [2,2,2] octane (100 mg/ml in PBS; Sigma) to prevent quenching of fluorescence, and mounted with glycergel (DABCO Corp., Carpinteria, CA). Fluorescence was observed with the use of a LEICA TCS spectral equipped with a DMR inverted microscope and a 63/1.4 objective. Image processing was performed with the use of the on-line "Scan Ware" software. Numeric images were processed with the use of Scion Image and Photoshop 5.0 softwares.

Metabolic Labeling and Immunoprecipitation

For metabolic labeling, the basolateral medium was replaced by methionine/cysteine-free medium (ICN Pharmaceuticals France, Orsay) containing 150 μCi of [^{35}S]methionine/cysteine (Promix; Amersham Pharmacia, Les Ulis, France) supplemented with 5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM sodium butyrate. Cells were labeled for 16 h, cooled on ice, and then biotinylated either on the apical or basolateral cell surface with freshly prepared 0.5 mg/ml NHS-LC-biotin (Pierce/Interchim, Lyon, France), twice for 20 min (Le Bivic *et al.*, 1989; Zurzolo *et al.*, 1992). Cells were lysed in the presence of protease inhibitors as described previously (Schell *et al.*, 1992; Scott *et al.*, 1997), centrifuged at 11,600 $\times$ *g* for 15 min at 4°C, and the proteins were immunoprecipitated. For immunoprecipitation of constructs bearing the ectodomain of NPP1, 70 μl Protein A-Sepharose beads (1 mg protein A/ml; Amersham, Pharmacia) were preincubated for 4 h at 4°C with a rabbit anti-mouse antibody (ICN; 4.5 μg/70 μl Protein A-Sepharose beads); then the beads were incubated for 4 h at 4°C with IR518 ascites diluted 1:250 and finally with the cell lysate for 4 h at 4°C. For immunoprecipitation of constructs bearing the ectodomain of NPP3, 70 μl Protein A-Sepharose beads were incubated for 4 h at 4°C directly with B10 ascites diluted 1:250 and incubated with the lysates for 4 h at 4°C. After washing, beads were boiled with 20 μl of 10% SDS for 5 min to elute the protein, and the beads were removed by centrifugation. The supernatant was diluted with 2 $\times$ 250 μl lysis buffer containing Triton X-100. Biotinylated material was recovered by adding 50 μl streptavidin beads (Pierce/Interchim) for 2 h at 4°C, followed by washing to remove unbound protein. The beads were then processed for SDS-PAGE and fluorography as described previously (Schell *et al.*, 1992; Scott *et al.*, 1997; Rajho Meerson *et al.*, 2000). Quantification of the bands was performed after scanning the gels with an Arcus II densitometric scanner (Agfa, Leverkusen, Germany), with the use of Scion Image software.

Endocytosis Assay

Endocytosis of NPP1 and NPP1/MLL was analyzed by studying the internalization of mAb IR 518 anti-mouse NPP1 at the cell surface of MDCK cells transfected with either construct. IR 518 diluted 1:400 was added either to the apical or basolateral medium of filters grown cells and incubated for 1 h at 37°C. After three washes with PBS to remove unbound antibody, cells were fixed with 2% paraformaldehyde for 20 min, permeabilized with 0.075% saponin for 15 min, and stained with the secondary FITC-conjugated antibody. Filters were then mounted with glycergel, and fluorescence was observed as described above. The absence of nonspecific uptake of antibody was verified by incubating the cells with an irrelevant mAb of the same isotype in the same conditions.

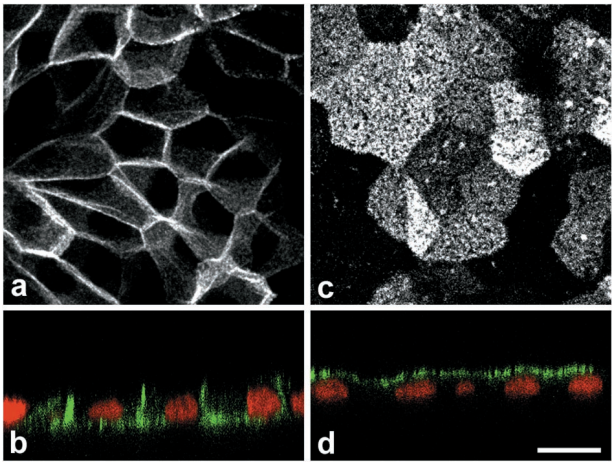

Figure 2. Confocal microscopic examination of the expression of NPP1 and NPP3 wild-types in polarized MDCK cells. MDCK cells stably transfected with NPP1 or NPP3 wild-types were grown on Transwell filters units, fixed, permeabilized and stained with mAb IR518 anti-mouse NPP1 (a and b) or with mAB B10 anti-rat NPP3 (c and d) and secondary FITC-conjugated antibody as described in MATERIALS AND METHODS. NPP1/WT is mainly restricted to the basolateral surface (a and b), whereas NPP3/WT is expressed at the apical surface (c and d). (a and c) Projections of all 0.5-μm xy focal sections taken throughout the height of the cells. (b and d) xz focal sections perpendicular to the xy focal sections; nuclei are stained in red with propidium iodide. Bar, 20 μm.

RESULTS

NPP1 Is Expressed at the Basolateral Surface of MDCK Cells

We first analyzed the localization of wild-type NPP1 in transfected cell populations. MDCK cells stably expressing NPP1 were identified by indirect immunofluorescence with the use of mAb IR518, a mAb specific for an extracellular epitope of the mouse protein. To characterize the steady-state distribution of NPP1, MDCK cells grown on Transwell filters were fixed, permeabilized, and stained with the anti-NPP1 antibody. Analysis by confocal microscopy showed a strong labeling of the basolateral membrane with a reticular pattern characteristic of basolateral antigens (Figure 2a). Transverse (xz) sections confirmed that NPP1 was exclusively expressed at the basal and lateral surfaces of transfected MDCK cells (Figure 2b). By contrast, rat NPP3, which is apically located in epithelial cells (Scott *et al.*, 1997), was exclusively expressed at the apical surface in transfected MDCK cells (Figure 2c). Confocal vertical (xz) sections showed a linear labeling above the nucleus, characteristic of apical expression (Figure 2d). To confirm the polarized surface expression of these proteins observed by immunofluorescence, we quantified the steady-state distribution at the apical and basolateral surfaces by metabolic labeling and

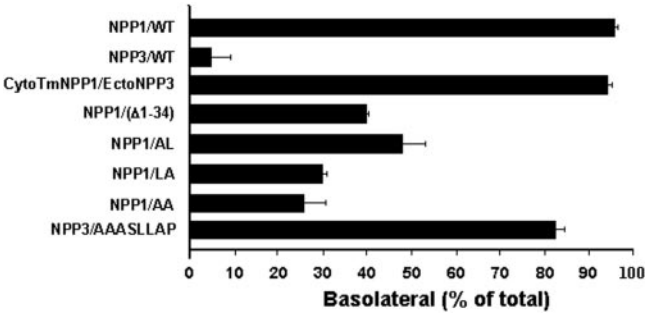

Figure 3. Quantification of the steady-state distribution of NPP1 and NPP3 wild-types, and different constructs. MDCK cells expressing NPP1/WT, NPP3/WT, CytoTmNPP1/EctoNPP3, NPP1/Δ1-34, NPP1/LA, NPP1/AL, NPP1/AA, and NPP3/AAASLLAP were grown to confluence on Transwell filters, labeled for 16 h with [^{35}S]methionine/cysteine, and biotinylated either from the apical or from the basolateral sides. Proteins were immunoprecipitated, then the biotinylated proteins were recovered by streptavidin precipitation and analyzed by electrophoresis and fluorography. Results are means ± SD of three to five determinations.

surface biotinylation. Filter-grown cells were labeled for 16 h with [^{35}S]methionine/cysteine and cooled on ice, and biotin was added either to the apical or basolateral surface. After lysing the cells, proteins were immunoprecipitated with anti-NPP1 or anti-NPP3 antibody and then precipitated with streptavidin and analyzed by 7.5% SDS-PAGE and fluorography. Approximately 98% of wild-type NPP1 was expressed at the basolateral membrane, whereas 95% of wild-type NPP3 was at the apical membrane (Figure 3).

The Cytoplasmic Domain Is Involved in Basolateral Targeting of NPP1

To determine in which domain of NPP1 the basolateral targeting signal is located, several chimeras were generated. We constructed chimeras in which the ectodomain of NPP1 was swapped by GFP or by the ectodomain of NPP3 (Figure 1B). GFP is a soluble protein that has no potential glycosylation sites and no targeting signal. A third chimera, CytoNPP1/TmEctoNPP3, was made of the cytoplasmic domain of NPP1 fused with the transmembrane and extracellular domains of NPP3. The fourth, CytoNPP3/TmEctoNPP1 contained the cytoplasmic domain of NPP3 and the transmembrane and extracellular domains of NPP1 (Figure 1B). When the ectodomain of NPP1 was substituted by GFP or by the ectodomain of NPP3, the chimeras were still expressed at the basolateral membrane, as for wild-type NPP1 (Figure 4, a–d). Biochemical analysis of these chimeras confirmed the confocal microscopy data in that >95% of the CytoTmNPP1/EctoNPP3 chimera was detected at the basolateral plasma membrane (Figure 3). Similarly, the CytoNPP1/TmEctoNPP3 chimera was expressed at the basolateral membrane (Figure 4, e and f). All these constructs harbor the cytoplasmic domain of NPP1. On the contrary, the CytoNPP3/TmEctoNPP1, which bears the cytoplasmic domain of NPP3, was expressed at the apical surface (Figure 4, g and h). Thus, the cytoplasmic tail of NPP1 is both necessary and sufficient for basolateral targeting.

To further define which region in the cytoplasmic domain of NPP1 is required for basolateral sorting, we constructed two mutants deleted of various parts of the N-terminal tail of the protein (Figure 1C). The first mutant NPP1/Δ2–5 was deleted of the first four amino acids, which are conserved between human and mouse (Belli and Goding, 1994). The second mutant NPP1/Δ1-34 was deleted of the first 34 amino acids, making the protein begin at the second methionine. The four amino acid–deleted mutant NPP1/Δ2-5 was entirely expressed at the basal and lateral membranes as wild-type NPP1 (Figure 5, a and b). By contrast, NPP1/Δ1-34 was mainly expressed at the apical membrane, although some labeling was also detectable at the middle and basal levels of the cells (Figure 5, e–g). Quantification by surface biotinylation indicated that 60% of NPP1/Δ1-34 was expressed at the apical surface and only 40% at the basolateral membrane (Figure 3). Therefore, the basolateral sorting information is likely to reside within amino acids 6–34.

Basolateral Sorting of NPP1 Requires a Di-leucine Motif

Within this 30 amino acid sequence, 2 leucines are found at positions 31 and 32. A di-leucine motif has been shown to be responsible for basolateral targeting of the macrophage IgG Fc receptor FcRII-B2 (Hunziker and Fumey, 1994). We therefore investigated the role of the di-leucine motif in targeting NPP1 to the basolateral surface. We made single or double mutations of the leucines into alanines, thus generating three mutants: NPP1/AL, NPP1/LA, and NPP1/AA (Figure

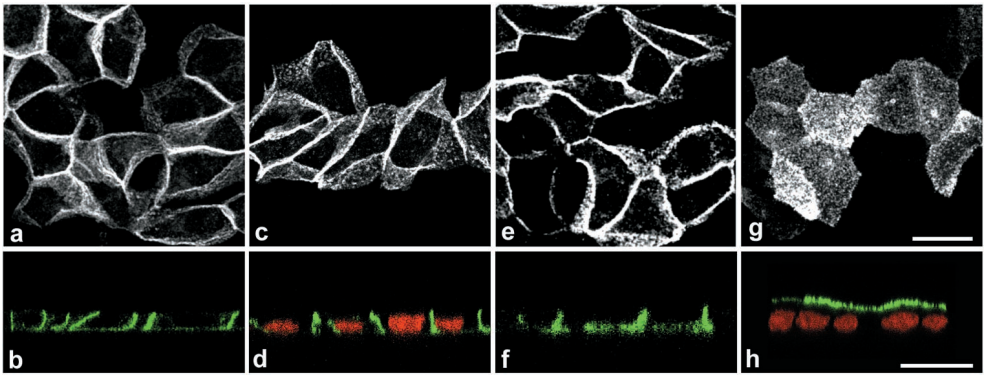

Figure 4. The cytoplasmic domain of NPP1/WT is necessary and sufficient for basolateral sorting. Cells were processed for indirect immunofluorescence microscopy as described in the legend of Figure 2 and analyzed by projections of all 0.5-μm confocal xy sections taken through the height of the cells (a, c, e, and g), and xz sections (b, d, f, and h) are represented for each chimera. CytoTmNPP1/EctoGFP (a and b), CytoTmNPP1/EctoNPP3 (c and d), CytoNPP1/TmEctoNPP3 (e and f), and CytoNPP3/TmEctoNPP1 (g and h). Bars, 20 μm.

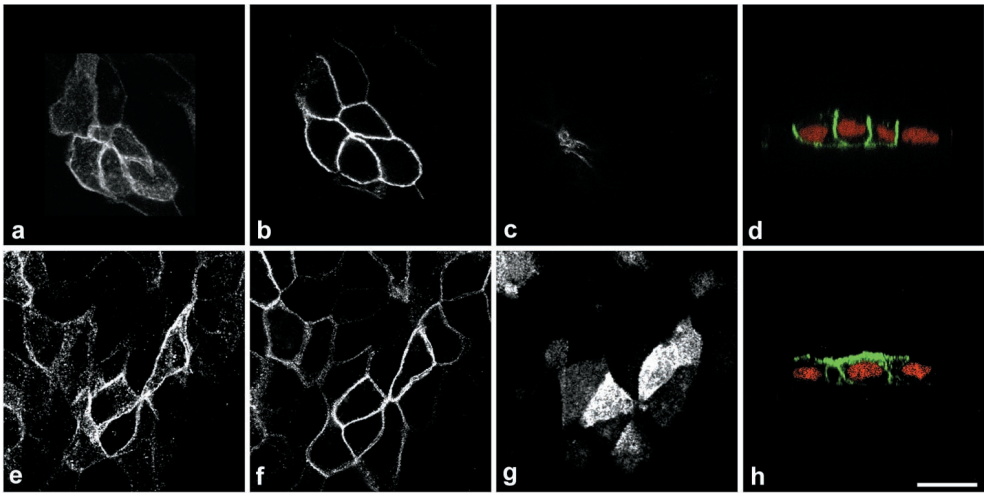

Figure 5. The basolateral signal is localized within amino acids 6–34. Localization of truncated protein NPP1/Δ2-5 (a–d) and NPP1/Δ1-34 (e–h) was analyzed by confocal laser scanning microscopy. Each picture is a sum of three 0.5-μm confocal microscopy xy sections from the bottom (a and e), middle (b and f), and top (c and g) of the cells. (d and h) xz sections. Bar, 20 μm.

1E). Confocal microscopy showed that all three mutants were expressed both at the apical and basolateral surfaces (Figure 6). Biochemical results showed that 60 and 70%, respectively, of the point mutants NPP1/AL and NPP1/LA and ~75% of the mutant NPP1/AA were detected at the apical surface (Figure 3). Thus these results identify the di-leucine motif as critical for basolateral sorting of NPP1.

To test whether the di-leucine motif is sufficient to constitute a basolateral targeting signal, we deleted the first 30 amino acids, so that NPP1 would begin immediately at the di-leucine motif (NPP1/MLL; Figure 1D). NPP1/MLL was found not only at the basal and lateral membranes of MDCK cells but also in cytoplasmic vesicles (Figure 7, a–d). This result indicates that the removal of the amino acids preceding the di-leucine motif targets NPP1 to an intracellular compartment and suggests that amino acids located upstream the di-leucine motif are probably required for basolateral targeting. Similar truncations with mutations of ei-

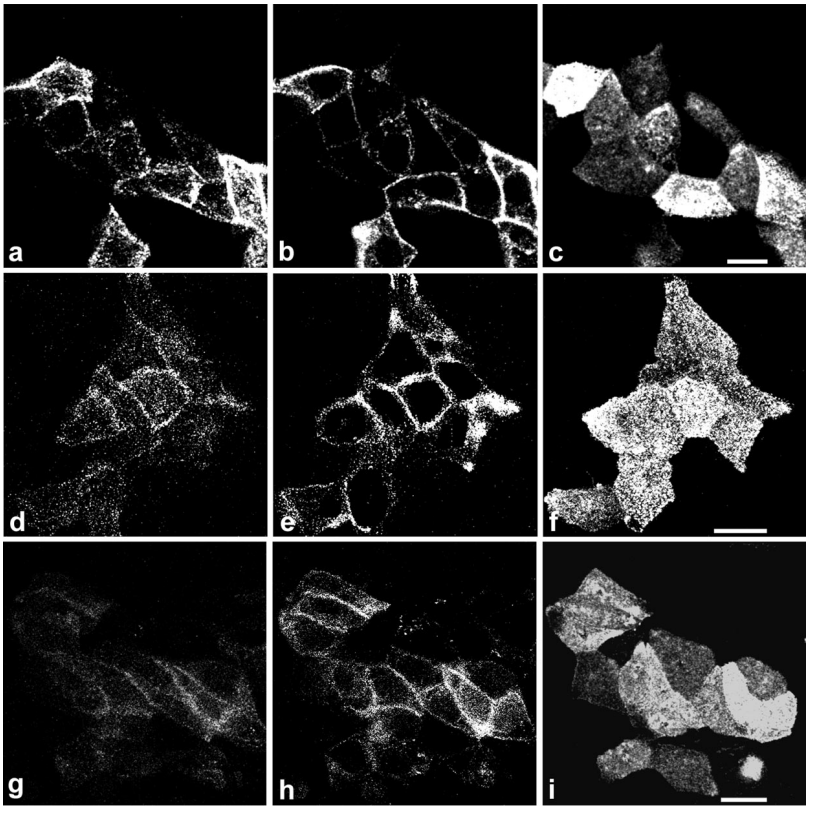

Figure 6. Basolateral sorting requires a di-leucine motif. Expression of point mutants NPP1/AL (a–c), NPP1/LA (d–f), and NPP1/AA (g–i) in MDCK cells. Each pictures is a sum of three 0.5-μm confocal microscopy xy sections from the bottom (a, d, and g), middle (b, e, and h), and top (c, f, and i) of the cells. Bars, 20 μm.

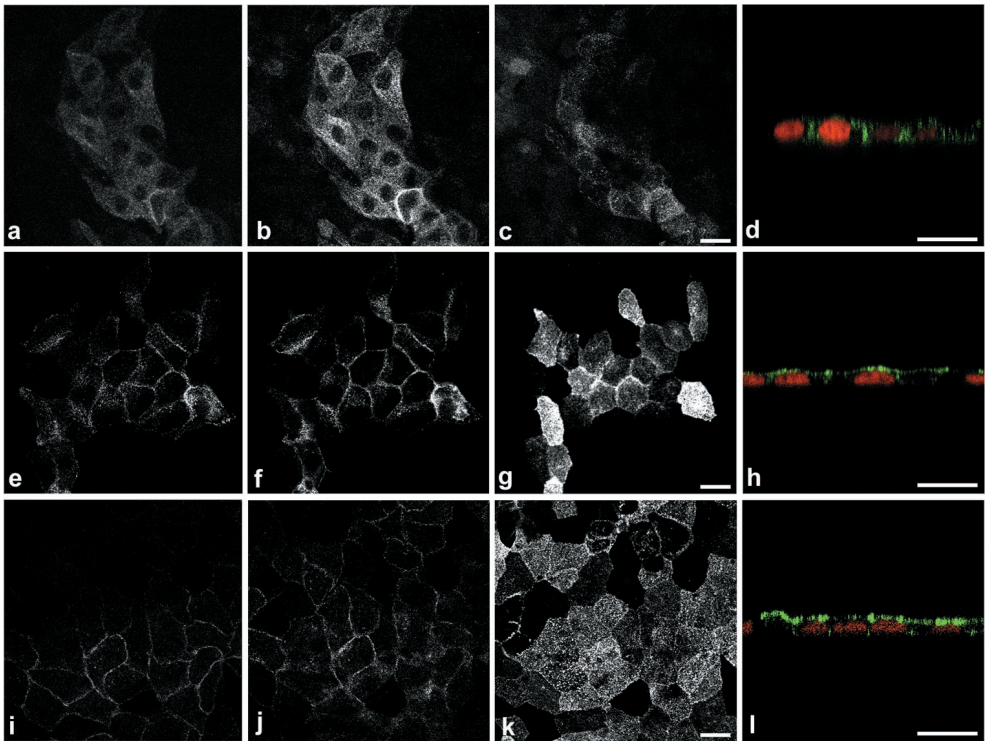

Figure 7. Truncation of the tail upstream the di-leucine motif unmasks an endocytosis signal. Expression of NPP1/MLL (a–d), NPP1/MAL (e–h), and NPP1/MLA (i–l). Each panel is a sum of three 0.5-μm confocal microscopy xy sections from the bottom (a, e, and i), middle (b, f, and j), and top (c, g, and k) of the cells. (d, h, and l) xz sections. Bars, 20 μm.

ther leucine into alanine (constructs NPP1/MAL and NPP1/MLA; Figure 1D), were seen exclusively at the cell surface, both at the apical and basolateral plasma membrane (Figure 7, e–l), indicating that truncation by itself does not cause intracellular retention.

The Basolateral Signal of NPP1 Does Not Mediate Endocytosis but Can Be Converted to an Internalization Signal

Many basolateral targeting signals have been shown to coincide or overlap with endocytosis signals (Matter and Mellman, 1994). At steady state, NPP1 was almost entirely located on the cell surface and was not found intracellularly, in contrast to the mutant NPP1/MLL. Therefore we tested endocytosis of NPP1 and NPP1/MLL by allowing internalization of mAb anti-NPP1. The antibody was added to either the apical or basolateral surfaces of filter-grown cells for 1 h at 37°C. Then cells were fixed with paraformaldehyde, permeabilized with saponin, and incubated for 1 h with a labeled secondary antibody. Confocal microscopy analysis showed that NPP1/WT was not substantially internalized. When the mAb was added to the apical surface of filter-grown cells, no labeling was visible; when it was added to the basolateral surface, a strong labeling of the lateral membranes was visible, but no significant intracellular labeling was detected (Figure 8a). In contrast, when the same experiments were performed in MDCK cells transfected with NPP1/MLL, a strong labeling of cytoplasmic vesicles was

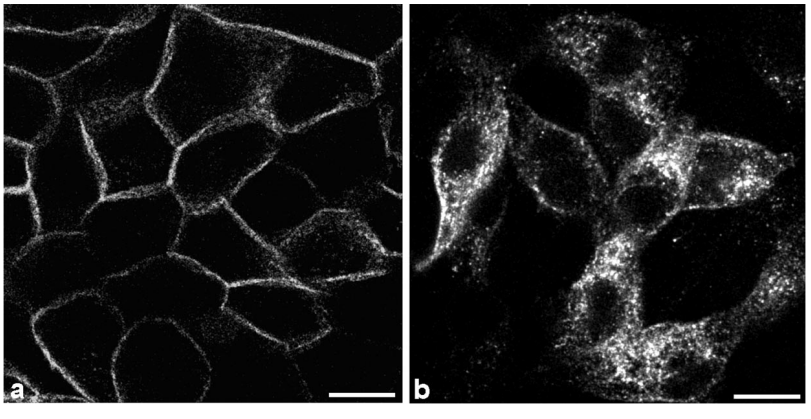

Figure 8. Morphological analysis of NPP1/WT and NPP1/MLL endocytosis. Filter-grown cells stably transfected with NPP1/WT (a) or NPP1/MLL (b) were incubated for 1 h at 37°C with mAb IR 518 anti-mouse NPP1 added to the basolateral medium, as described in MATERIALS AND METHODS. After washing, fixation, and permeabilization, the antibody was visualized with a secondary FITC-conjugated antibody. (a) and (b) Single 0.5-μm xy sections taken from the middle of the cells. Bars, 20 μm.

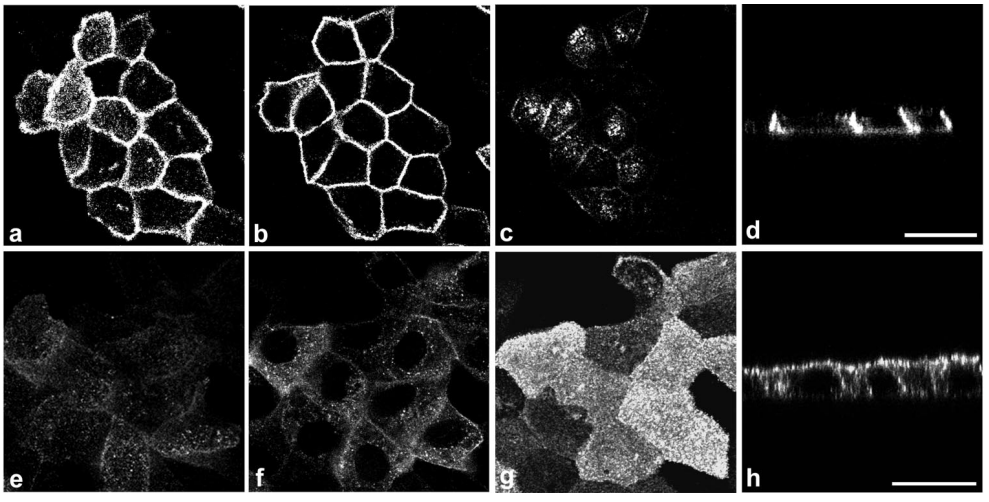

Figure 9. The sequence AAASLLAP but not the sequence LLAP is able to redirect NPP3 to the basolateral surface. MDCK cells stably transfected with NPP3/AAASLLAP or with NPP3/LLAP were studied by confocal microscopy with the use of a mAb anti-NPP3 as described in the legend of Figure 2. Each picture is a sum of three 0.5-μm confocal microscopy xy focal sections taken at the bottom (a), middle (b), and top (c) of the cells. (d) An xz section. Bar, 20 μm.

observed (Figure 8b), showing that a detectable fraction of NPP1/MLL was internalized. Internalization was only detected when the antibody was added in the basolateral medium, indicating that NPP1/MLL was first delivered to the basolateral cell surface, where it could bind and internalize the antibody.

The Basolateral Signal of NPP1 Targets NPP3 to the Basolateral Surface

To establish unequivocally that the di-leucine motif of NPP1 is a basolateral signal, it is necessary to demonstrate that this motif is able to redirect a nonbasolateral protein to the basolateral domain. We thus grafted the di-leucine motif of NPP1 and a few surrounding amino acids onto the cytoplasmic domain (Figure 1F) of the apically expressed protein NPP3 (see Figure 2c). The sequence AAASLLAP was chosen because it was entirely conserved within the cytoplasmic tail of human and mouse NPP1. As shown in Figure 9, a–c, the chimera NPP3/AAASLLAP was expressed at the basal and middle levels of MDCK cells; confocal vertical section confirmed that the chimeric construct was virtually exclusively expressed on the lateral membranes (Figure 9d). As shown in Figure 3, >80% of the chimera was delivered to the basolateral membrane. Thus, the short sequence AAASLLAP encodes an effective basolateral targeting signal that is dominant over the apical targeting signal of NPP3.

Like NPP1/WT, NPP3/AAASLLAP was not found intracellularly. To see if the AAASLLAP signal could also be converted to an endocytosis signal in the context of NPP3, the sequence LLAP was added to the cytoplasmic tail of NPP3 immediately after the first methionine, thus reproducing the N-terminal sequence of NPP1/MLL (Figure 1D). Some intracellular labeling was indeed observed (Figure 9f). However, the construct NPP3/LLAP was mainly found at the apical membrane, with only little basolateral staining (Figure 9, e–h).

Amino Acids Flanking the di-leucine Motif Are Required for Exclusive Basolateral Targeting

To define more precisely the amino acids environment required for the di-leucine motif to mediate exclusive basolateral targeting or intracellular targeting, we made an additional series of mutants in which the flanking upstream amino acids were changed. A deletion mutant was constructed to remove amino acids preceding the di-leucine motif. The sequence SPAAAS (amino acids 25–30) was removed because deletion of AAAS only would again bring a serine close to the di-leucine (Figure 1C). This construct, NPP1/Δ25-30, was targeted to an intracellular compartment (Figure 10a), like the mutant NPP1/MLL (cf. Figure 7b). Likewise, endocytosis experiments indicated that the mutant NPP1//Δ25-30 was internalized from the basolateral surface. Mutation of Ala28 into Gly28 did not change basolateral expression (Figure 10b). Mutations of Ser30 into Ala or Asp produced some intracellular localization in addition to the strong basolateral staining (Figure 10, c and d). However, only little internalization was observed in endocytosis experiments.

DISCUSSION

We have previously shown that the ecto-nucleotide pyrophosphatase/phosphodiesterase NPP1 has a restricted basolateral localization in rat hepatocytes, whereas NPP3, another member of the family is apically located (Scott *et al.*, 1997). The same polarized distributions were observed in MDCK cells transfected with mouse NPP1 or rat NPP3. Therefore, transfected MDCK cells are suitable models in order to study the mechanisms for targeting NPPases to their respective plasma membrane locations in epithelial cells.

The mechanisms for sorting and targeting proteins to the apical and basolateral surfaces are still only partially understood. Sorting of apical proteins may depend on specific interactions of the transmembrane domain or the glycosylphosphatidylinositol anchor with membrane microdomains (Simons and Ikonen, 1997) or on the presence of N- and O-glycans chains within the extracellular domain (Fiedler and Simons, 1995; Yeaman *et al.*, 1996; Gut *et al.*, 1998). However, the sorting signals of NPP3 are still not known. Recent investigations showed that neither glycosylation nor raft association seems to be the targeting mechanism (Rajho Meerson *et al.*, 2000).

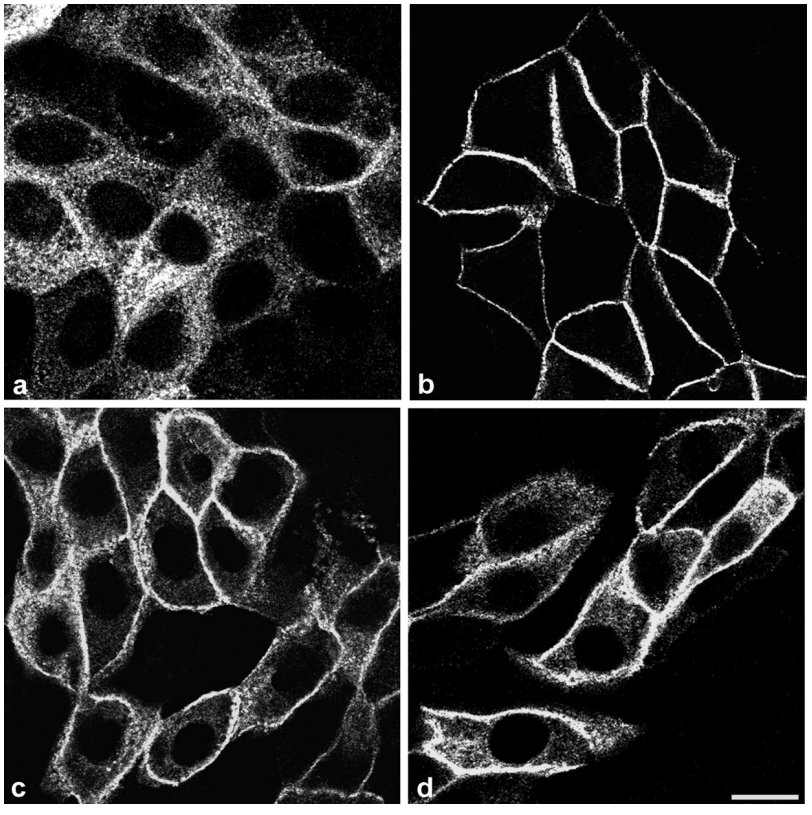

Figure 10. Flanking upstream amino acids are required for specific basolateral targeting of NPP1. Expression of deletion mutant NPP1/Δ25-30 (a) and point mutants NPP1/Gly (b), NPP1/Asp (c), and NPP1/Ala (d) were studied by confocal microscopy. Each panel is a single xy section taken from the middle of the cells. Bars, 20 μm.

Sorting of basolateral proteins has been shown to depend on short amino acid sequences located in the cytoplasmic tail (Aroeti *et al.*, 1998). Different classes of signals have been identified. The most frequent and best-characterized sequences depend on a critical tyrosine residue within a consensus sequence Tyr-X-X-Φ, where Φ is a bulky hydrophobic residue. Most of these tyrosine based-signals may also function as signals for rapid endocytosis. Less common signals include Leu-Leu and di-hydrophobic motifs or short sequences with no apparent consensus.

In this work, we were able to identify the basolateral targeting signal of NPP1 as a short cytoplasmic sequence comprised within the amino acid stretch AAASLLAP, which includes a conserved di-leucine motif. Mutations of either or both leucines largely redirected the protein to the apical surface. Furthermore, addition of the conserved sequence AAASLLAP to the apical protein NPP3 was sufficient to address the protein to the basolateral surface. This result indicates that the basolateral signal of NPP1 is dominant over the apical determinant of NPP3, a feature that has already been recognized for tyrosine-based basolateral signals and basolateral signals with no consensus sequence. In general, fusion of the cytoplasmic tails of basolateral proteins to the transmembrane and ecto-domains of apical proteins or introduction of a basolateral signal resulted in basolateral transport (Brewer and Roth, 1991; Casanova *et al.*, 1991; Matter *et al.*, 1992; Prill *et al.*, 1993; Thomas *et al.*, 1993; Kundu and Nayak, 1994; Thomas and Roth, 1994; Monlauzeur *et al.*, 1995; Lin *et al.*, 1997; Renold *et al.*, 2000).

Di-leucine motifs have been generally shown to target proteins to the endosomal/lysosomal system (Sandoval *et al.*, 1994). Only for the FcRII-2B receptor was a di-leucine motif involved in basolateral sorting (Hunziker and Fumey, 1994). The di-leucine of the FcRII-2B receptor also mediates entry into the endocytotic pathway, but in the case of NPP1, the di-leucine motif only mediates basolateral sorting because the protein is not rapidly endocytosed. A di-hydrophobic motif Leu-Val in the plasma membrane adhesion protein CD44 (Sheikh and Isacke, 1996) and a di-leucine motif in the Lutherian glycoprotein (El Nemer *et al.*, 1999) also appear to function solely as basolateral signals. However, because both these cases are cell-matrix adhesion molecules, they may be stabilized at the plasma membrane and thus be prevented from being internalized. The example of NPP1 suggests that some di-leucine motifs can function as basolateral signals only, whereas others are also able to target proteins to endosomes or lysosomes. Therefore, di-leucine-based signals would form a degenerate family of signals that function in targeting proteins to various intracellular compartments and to the basolateral domain of the plasma membrane, as do tyrosine-based signals (Marks *et al.*, 1997).

Because very few basolateral signals of the di-leucine type have been identified, the requirement for basolateral sorting versus endosomal targeting has not been much studied. An unexpected finding was that the deleted mutant NPP1/MLL beginning at the sequence MLL was mainly expressed intracellularly, suggesting that the basolateral signal was converted to an endocytosis signal. Indeed, endocytosis experiments demonstrated that the deleted mutant was internalized, whereas wild-type NPP1 was not, at least during the time of the assay. The fact that in this case very little

NPP1 was detectable at the basolateral surface, and yet endocytosis of bound antibody was rapid, suggests that the transit time at the basolateral membrane for this construct is short. However, we cannot exclude that some NPP1/MLL molecules were directly targeted from the trans-Golgi network to the intracellular compartment. Because internalization mainly occurred from the basolateral surface, the new motif probably functions both as a basolateral and endocytotic signal. However, contrary to the AAASLLAP motif, the MLL motif is not dominant over the apical determinants of NPP3, because NPP3/LLAP was mainly expressed at the apical surface, with only little basolateral and intracellular localization.

Analysis of environmental requirements for the di-leucine signal indicated that the amino acids AAAS preceding the two leucines are needed for specific basolateral targeting. Thus, NPP3/AAASLLAP was basolaterally expressed, whereas NPP1/Δ25-30 was mainly intracellular. However, point mutations did not allow us to identify crucial amino acids. Mutation of the central alanine did not affect basolateral sorting, and mutation of the serine into alanine or aspartic acid only caused minimal intracellular localization. Analysis of the amino acid sequences flanking known basolateral di-leucine motifs did not reveal any homology with the AAASLLAP sequence. Therefore, the basolateral signal of NPP1 appears to be a unique motif for specific basolateral sorting.

Many di-leucine–based signals working in endocytosis require charged upstream amino acids to be functional (Pond *et al.*, 1995; Simmen *et al.*, 1999; Sandoval *et al.*, 2000; Shewan *et al.*, 2000). This does not appear to be the case for the engineered endocytotic signals of NPP1/MLL and NPP1/Δ25-30 in which it seems to be the absence of the AAAS sequence that allows the constructs to be internalized. The sequence AAAS may act either as a retention signal at the plasma membrane or prevent entry of the protein into the endocytotic pathway.

The functional diversity of leucine-based motifs suggests that a family of receptors is able to recognize and discriminate between resembling signals. Furthermore, the similarity between basolateral and endocytotic signals suggests that these receptors may be clathrin adaptor protein (AP) complexes. Four different AP complexes have been identified (Kirchhausen, 1999). AP-1 and AP-2 complexes bind to the cytoplasmic tail of proteins and subsequently recruit clathrin, at the Golgi apparatus and plasma membrane, respectively. AP complexes have been shown to bind both to tyrosine and di-leucine–based motifs. Binding of tyrosine-based motifs to the μ chains of AP complexes is well established and characterized (Bonifacino and Dell'Angelica, 1999), but there is some controversy as to whether di-leucine motifs interact with the μ or β chains of AP complexes (Ohno *et al.*, 1995; Heilker *et al.*, 1996; Bremnes *et al.*, 1998; Rapoport *et al.*, 1998; Rodionov and Bakke, 1998; Hofmann *et al.*, 1999).

Involvement of the AP-1 complex in basolateral sorting of the pIg receptor is supported by coimmunoprecipitation experiments (Orzech *et al.*, 1999). Furthermore, a new μ subunit of the AP-1 complex, termed μ1B, has been identified as a specific subunit expressed by epithelial cells (Ohno *et al.*, 1999) and involved in basolateral sorting (Folsch *et al.*, 1999). However, it is not clear whether the AP-1 complex is able to bind all types of basolateral signals and if it is the only mechanism for basolateral sorting. For instance, the FcRII-2B receptor, whose basolateral signal is a di-leucine, is correctly targeted to the basolateral surface of LLC-PK1 cells (Roush *et al.*, 1998), although these cells do not express μ1B (Folsch *et al.*, 1999) and missort many basolateral proteins to the apical surface. The recent discovery of a new family of monomeric adaptors with homology to the γ subunit of the AP-1 complex (Dell'Angelica *et al.*, 2000; Hirst *et al.*, 2000) shows that several mechanisms are involved in the sorting of proteins at the trans-Golgi network. Further work will be needed to precise whether interaction with a specific subunit of the AP-1 complex or another mechanism is involved in the sorting of NPP1 and other basolateral proteins with di-leucine motifs and how the flanking amino acids may change the relative affinity of the signal to different binding partners.

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